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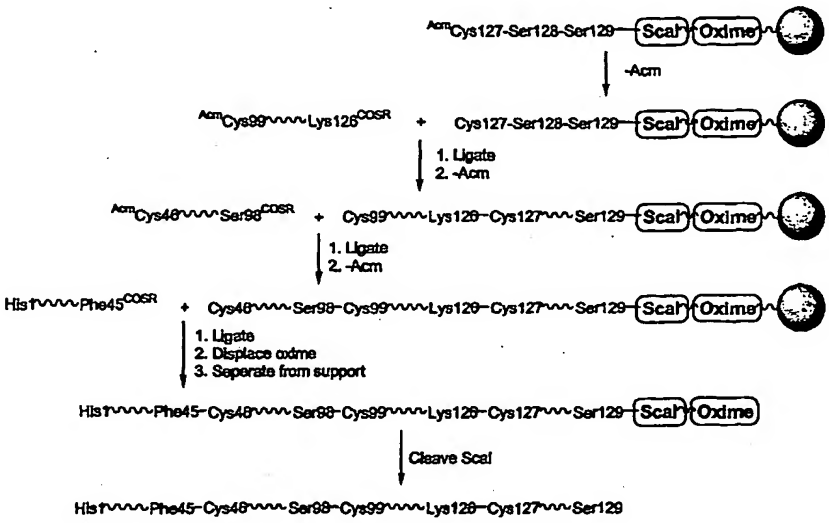
(54) Title: DISPLACEABLE LINKER SOLID PHASE CHEMICAL LIGATION



(57) Abstract: The invention is directed to methods and compositions for aqueous-compatible solid phase chemical ligation. The methods and compositions of the invention involve the chemical ligation of first and second polymers, where the first polymer is attached to a support through a displaceable linker. The displaceable linker can be cleaved or otherwise displaced under aqueous conditions compatible with subsequent removal of the ligation product from the solid support. The methods and compositions of the invention are particularly useful for ligation of peptides and polypeptides on a solid support. The ligation system of the invention is applicable to a wide variety of molecules, and thus can be exploited to generate ligated polymers including peptides, polypeptides and other amino acid containing polymers.

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DISPLACEABLE LINKER SOLID PHASE CHEMICAL LIGATION

CROSS-REFERENCES TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Patent Application No. 60/473,294, filed May 22, 2003, the content of which is incorporated herein by reference.

STATEMENT AS TO RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0002] NOT APPLICABLE

REFERENCE TO A "SEQUENCE LISTING," A TABLE, OR A COMPUTER PROGRAM LISTING APPENDIX SUBMITTED ON A COMPACT DISK.

[0003] NOT APPLICABLE

INTRODUCTION

Technical Field

[0004] The invention relates to methods and compositions for aqueous-compatible solid phase chemical ligation of a wide range of polymer molecules, including peptides, polypeptides, nucleic acids, lipids, carbohydrates, synthetic polymers and other molecules.

BACKGROUND OF THE INVENTION

[0005] Chemical ligation involves the formation of a selective covalent linkage between a first chemical component and a second chemical component. Unique, mutually reactive, functional groups present on the first and second components can be used to render the ligation reaction chemoselective. For example, the chemical ligation of peptides and polypeptides involves the chemoselective reaction of peptide or polypeptide segments bearing compatible unique, mutually reactive, C-terminal and N-terminal amino acid residues. The ligation components can be partially or fully unprotected, rendering them useful for reactions in aqueous-based systems. Several different chemistries have been utilized for this purpose, examples of which include amide-forming chemical ligation (Dawson, *et al., Science* (1994) 266:776-779; Kent, *et al.*, WO 96/34878; Kent, *et al.*, WO 98/28434; US Patent No. 6,307,018; and US Patent No. 6,184,344).

[0006] The principles of aqueous-based chemical ligation have recently been adapted for the chemical synthesis of peptides and polypeptides on a solid support (Canne, *et al., J. Amer. Chem. Soc.* (1999) 121:8720-8727; and US Patent No. 6,326,468). In this approach, a first substrate is attached to the solid support through a cleavable handle or linker. A second substrate is then ligated in aqueous medium to the first substrate on the solid support. Cleavage of the linker allows the reaction product to be released from the solid phase.

[0007] While aqueous-based chemical ligation on a solid support has proven very useful, improvements in synthesis, recovery, purity, yield and overall robustness are needed. The present invention addresses these and other needs.

Literature

[0008] Aqueous-based chemical ligation on a solid support is discussed in the following references: Canne, *et al., J. Amer. Chem. Soc.* (1999) 121:8720-8727; US Pat, No. 6,326,468; and Brik, *et al., J. Org. Chem.* (2000) 65:3829-3835.

[0009] Aqueous-based chemical ligation in solution is discussed in the following references: Dawson, *et al., Science* (1994) 266:776-779; WO 96/34878; WO 98/28434; Rose, *et al., J. Amer. Chem. Soc.* (1994) 116:30-33; Schnölzer, *et al., Science* (1992) 256:221-225; Englebretsen, *et al., Tet. Letts.* (1995) 36(48):8871-8874; Gaertner, *et al., Bioconj. Chem.* (1994) 5(4):333-338; Zhang, *et al., Proc. Natl. Acad. Sci.* (1998) 95(16):9184-9189; WO 95/00846; Yan, *et al., J. Am. Chem. Soc.* (2001) 123:526-533, Gieselnan, *et al., Org. Lett.* (2001) 3(9):1331-1334; Saxon, E., *et al., Org. Lett.* (2000) 2:2141-2143.

[0010] Linker systems and supports are discussed in the following references: "Advanced Chemtech Handbook of Combinatorial & Solid Phase Organic Chemistry," W.D. Bennet, J.W. Christensen, L.K. Hamaker, M.L. Peterson, M.R. Rhodes, and H.H. Saneii, Eds., Advanced Chemtech, 1998, and elsewhere (See, e.g., G.B. Fields *et al., Synthetic Peptides: A User's Guide*, 1990, 77-183, G.A. Grant, Ed., W.H. Freeman and Co., New York; NovaBiochem Catalog, 2000; "Synthetic Peptides, A User's Guide," G.A. Grant, Ed., W.H. Freeman & Company, New York, NY, 1992; "Principles of Peptide Synthesis, 2nd ed.," M. Bodanszky, Ed., Springer-Verlag, 1993; "The Practice of Peptide Synthesis, 2nd ed.," M. Bodanszky and A. Bodanszky, Eds., Springer-Verlag, 1994; "Fmoc Solid Phase Peptide Synthesis, A Practical Approach", W.C. Chan and P.D. White, Eds., Oxford Press, 2000; and Bioconjugate Techniques, Greg T. Hermanson, Academic Press, 1996.)

BRIEF SUMMARY OF THE INVENTION

[0011] The invention is directed to methods and compositions related to aqueous-compatible solid phase chemical ligation. The aqueous-compatible solid phase chemical ligation method of the invention comprises: (a) ligating under aqueous conditions a first polymer to a second polymer to form a ligation product bound to an aqueous-compatible support, the first and second polymers having mutually reactive chemical groups capable of chemoselective chemical ligation, the first polymer being attached to the support through a linker system comprising a displaceable linker; (b) releasing the ligation product from the support under conditions that displace the displaceable linker; and (c) separating the ligation product from the support.

[0012] The method of the invention preferably employs a multi-detachable linker system for tailoring the newly generated terminal end of the ligation product following displacement and separation from the support. This method involves: (a) ligating under aqueous conditions a first polymer to a second polymer to form a ligation product bound to an aqueous-compatible support, the first and second polymers having mutually reactive chemical groups capable of chemoselective chemical ligation, the first polymer being attached to the support through a linker system having a displaceable linker and a second linker that is cleavable under conditions orthogonal to displacement of the displaceable linker, the second linker joining the first polymer to the displaceable linker; (b) displacing the displaceable linker so as to release the ligation product from the support; (c) separating the ligation product from the support; and (d) cleaving the second linker so as to remove the second linker from the ligation product.

[0013] The invention is further directed to compositions for aqueous-compatible solid phase chemical ligation. The compositions include, in one embodiment, a support-bound polymer having (i) a first end attached to a support through a linker system that includes a displaceable linker, and (ii) a second end bearing a protected or unprotected chemoselective reactive group capable of chemoselective chemical ligation with a mutually reactive chemoselective reactive group. The displaceable linkers of such compositions include chemically and/or enzymatically displaceable linkers. In a specific embodiment, the linker system of such compositions may include a chemically displaceable linker such as a hydrazone linker, a diol linker, a photolabile linker, a reducible linker, or a metal chelator linker. In another specific embodiment, the linker system of such compositions comprises an

enzymatically displaceable linker that is a hydrolytic enzyme, such as an esterase, a lipase, an endoprotease, an endoglyconase, or a nucleic acid restriction enzyme.

[0014] The invention also is directed to a composition that includes a reaction product of a solid phase chemical ligation reaction, where the reaction product comprises a partially or fully unprotected polymer that is substantially pure and free of a solid support, and where the polymer includes a first end attached to a displaceable linker through a second linker that is cleavable under conditions orthogonal to the displaceable linker. This composition may also include water and an excipient, such as a buffer, ligation catalyst, denaturant, lipid, detergent and chaotrope. The composition may also be a lyophilized powder. In certain embodiments, the polymer of such composition is substantially monodisperse (i.e., predominantly a single molecular species of defined covalent structure, in contrast to heterodisperse composition that contains a chemical ligation product composed of a range molecular species each having a different covalent structure).

[0015] Kits also are provided. The kits include one or more containers having deposited therein a composition of the invention, a protocol for carrying out a method of the invention, or a combination of both.

[0016] The polymer components employed in the solid phase chemical ligation methods of the invention can include a wide range of chemical moieties, including amino acids, peptides, polypeptides, nucleic acids or other chemical moieties such as dyes, haptens, carbohydrates, lipids, biocompatible polymers or other polymers and the like. The solid phase chemical ligation method of the invention is robust, providing faster access to polymer compounds in surprisingly high and pure yields, and increasing the average size of the polymers that can be synthesized made using standard chemical ligation approaches. Moreover, the solid phase chemical ligation methods and compositions of the invention expand the utility of chemical ligation to multi-component ligation schemes, such as when producing a polypeptide involving multiple ligation strategies, such as a three or more segment ligation scheme or convergent ligation synthesis schemes.

[0017] There are several additional advantages provided by the invention. The solid phase chemical ligation approach of the invention permits facile and improved recovery of the ligation product from the support, particularly for larger ligation products that would ordinarily adhere or otherwise bond to the support system when cleaved under harsh conditions used for non-displaceable linkers. Once a ligation product is recovered, the

displaceable linker can be removed or exploited for re-attachment to the same or different solid support. Also, as the displaceable linker permits recovery of the ligation product from the support under mild conditions, reaction substrates also may be linked to the support through a displaceable linker to any number of second cleavable linkers for subsequent tailoring of the new terminal end of the ligation product once released in solution. For example, the second linker can be any number of moieties removable under a variety of conditions, including linkers that are cleaved under strong acid, strong base, and the like. This is particularly useful since many such linkers can be used to specifically tailor terminal end(s) of the ligated polymers.

[0018] Further advantages include the ability to wash excess reagents and soluble side products away from the support thus making subsequent purification of the desired ligation product easier. Support bound substances also can be handled more easily and safely. Another advantage is that reactions that exhibit poor chemoselectivity can often be made more efficient by attachment of one or more of the components to the solid support, and combined in high concentrations to aid in driving the reactions forward. Accordingly, the methods and compositions of the invention greatly expand the scope of chemical ligation, and the starting, intermediate and final products of the invention find a wide range of uses.

BRIEF DESCRIPTION OF THE DRAWINGS

[0019] **Figure 1** illustrates a synthesis scheme for assembly of the Interleukin-4 (IL4) protein sequence utilizing aqueous-based solid phase chemical ligation with a displaceable oxime linker.

[0020] **Figure 2** shows Reverse-Phase High Performance Liquid Chromatography (RP-HPLC) chromatograms of purified full-length IL4 protein following release from the support. Panel **FIG.2A** depicts RP-HPLC of purified full-length IL4 with SCAL-Oxime linker still attached. Panel **FIG.2B** depicts RP-HPLC of purified full-length IL4 after removal of the SCAL-Oxime linker.

[0021] **Figure 3** illustrates a synthesis scheme for assembly of the IL4 protein sequence utilizing aqueous-based solid phase chemical ligation with a displaceable metal chelator linker.

[0022] **Figure 4** shows RP-HPLC chromatograms of purified full-length IL4 protein following release from the support. Panel **FIG.4A** depicts RP-HPLC of purified full-length

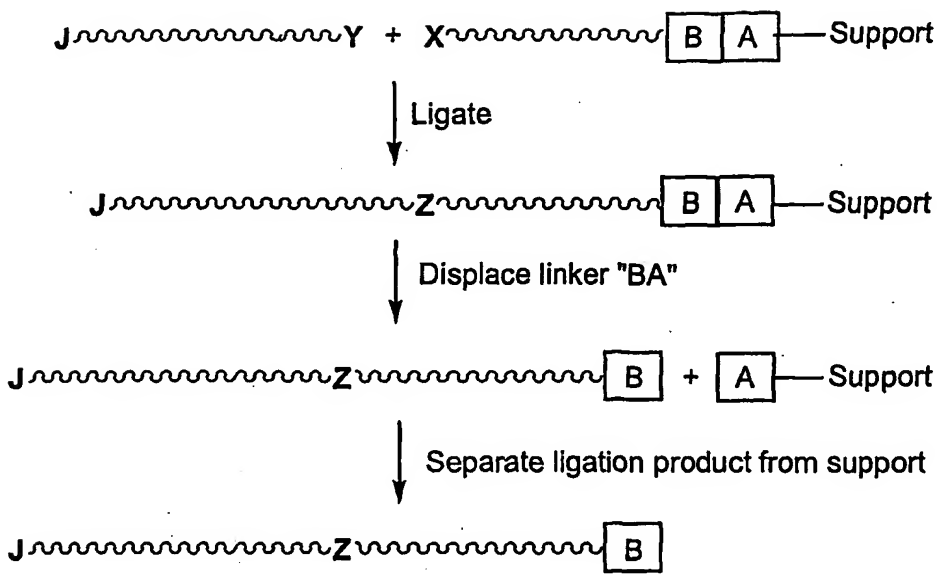
IL4 with Rink-HisTag linker still attached. Panel **FIG.4B** depicts RP-HPLC of purified full-length IL4 after removal of the Rink-HisTag linker.

DETAILED DESCRIPTION OF THE INVENTION

[0023] It has been discovered that while aqueous-based solid phase chemical ligation and subsequent cleavages are typically robust, there is a need to further improve separation of the released ligation product from the support. This is most apparent when pliable supports that swell in water are employed in combination with handles or linkers that require cleavage under relatively harsh chemical conditions. For example, linkers that require cleavage with strong acid or base can damage the support, or otherwise cause the product to adhere to the support, and increase the purification burden thereby making recovery of the desired product more difficult. Accordingly, the present invention is directed to new approaches that provide solutions to these and other problems.

[0024] In particular, the invention is directed to methods and compositions related to aqueous-compatible solid phase chemical ligation. In one embodiment, the invention is directed to a method for the aqueous-compatible solid phase chemical ligation of polymers. In this method, a composition is provided that includes a support-bound polymer having a first end attached to an aqueous-compatible support through a linker system having a displaceable linker, and a second end having a chemical group capable of chemoselective chemical ligation with a mutually reactive chemical group of a second polymer under aqueous conditions. The support-bound polymer is then contacted, under aqueous conditions, with a second polymer having a first end bearing a chemical group that is mutually reactive with the second end of the support-bound polymer, to form a ligation product having the first and second polymers bound to the aqueous-compatible support. The ligation product is then released from the support under conditions that displace or otherwise cleave the displaceable linker. Once released, the ligation product is separated from the support to yield a ligation product free of the support. This process is illustrated below in **Scheme 1**.

Scheme 1

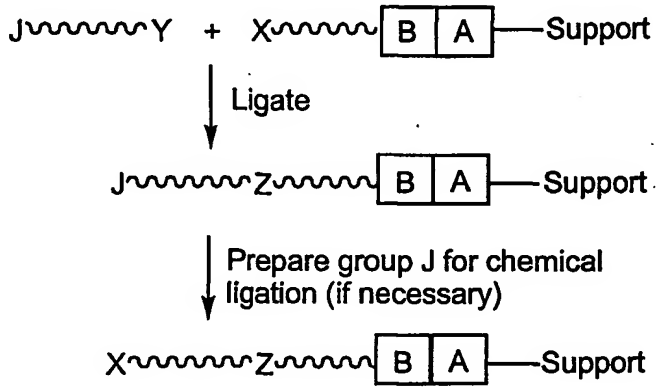


[0025] As shown in **Scheme 1**, the composition "X~BA~Support" represents a first polymer that is bound to an aqueous-compatible support, i.e., "Support" through a displaceable linker "BA", where the polymer portion is depicted by a wavy line. The first polymer also bears a chemoselective group "X" that is capable of chemical ligation. The second polymer "J~Y" is depicted as having a chemoselective group "Y" that is mutually compatible with, and capable of chemoselective chemical ligation with the X group on the first polymer. As groups X and Y are chemoselective for each other, they can react and form a covalent bond therein between in the presence or absence of other functional reactive groups, for instance, one or more side chain functional groups typical for unprotected amino acids. Thus, the first and second polymers may be partially or fully unprotected, depending on the intended end use. The second polymer also is shown with pendant group J, which may be present or absent, and represents an additional group that may be converted to a group that is capable of chemical ligation, or is a group capable of a chemical ligation reaction that is orthogonal to the chemical ligation reaction employed for joining the X and Y groups of the first and second polymers, respectively. Ligation of the first and second polymers results in the formation of a support-bound ligation product "J~Z~BA~Support" having ligation site "Z." Once ligated, the linker BA is displaced so as to release the ligation product "J~Z~B" from the support "A~Support." As shown in **Scheme 1**, the displacement reaction results in a ligation product having residual linker B, whereas the support has residual linker A attached thereto. Following release of the ligation product from the support by the displacement

reaction, the ligation product can then be separated from the solution containing the support. Although attachment of the linker moiety to the first polymer can be accomplished through a pendant terminal group of the polymer, as shown in **Scheme 1**, the linker moiety can be readily attached to a backbone group or side chain group (if present) on a polymer. For instance, while **Scheme 1** and other reaction schemes depicted herein illustrate attachment of such groups through or to a pendant terminal group on a polymer, a displaceable linker, linker system or component thereof may be attached to a backbone or side chain group on a polymer.

[0026] The first and second polymers may be prepared by a variety of methods. In a preferred embodiment, the first polymer attached to the support is formed by chemical ligation, as depicted in **Scheme 2**. The second polymer also may be the reaction product of a chemical ligation reaction.

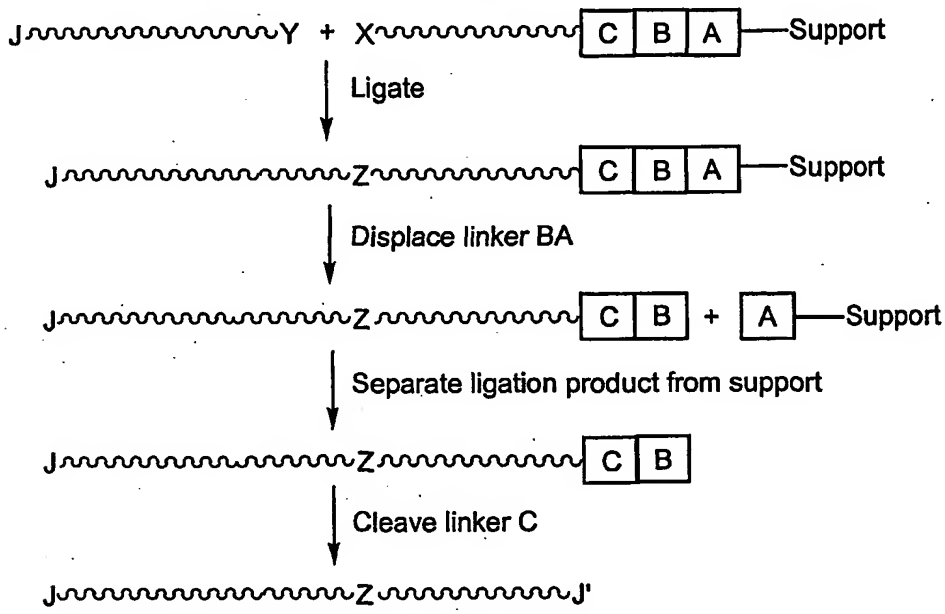
Scheme 2



[0027] The method of the invention preferably employs as part of the linker system a multi-detachable linker for tailoring the newly generated terminal end of the ligation product following its separation from the support. In this embodiment, a composition is provided that includes a support-bound polymer having a first end attached to an aqueous-compatible support through a linker system having a displaceable linker and a second linker that is cleavable under conditions orthogonal to displacement of the displaceable linker, where the second linker joins the polymer to the displaceable linker, and the displaceable linker is attached to the support. In addition to the linker system, the second end of the support-bound polymer includes a chemical group capable of chemoselective chemical ligation with a mutually reactive chemical group under aqueous conditions. For the ligation reaction, the support-bound polymer is contacted, under aqueous conditions, with a second polymer

having a first end bearing a chemical group that is mutually reactive with the second end of the support-bound polymer, to form a ligation product having the support-bound polymer and the second polymer. The ligation product is then released from the support under conditions that displace or otherwise cleave the displaceable linker. Once released, the ligation product is separated from the support to yield a ligation product free of the support. At this stage, the second linker may be removed or otherwise modified to tailor the newly generated terminal end of the ligation product free of the support. This process is depicted below in **Scheme 3**.

Scheme 3



[0028] As shown in **Scheme 3**, a multi-detachable linker system "CBA" is provided on the support-bound polymer, where BA represents a displaceable linker, and "C" represents the second linker. Displacement of linker BA generates a ligation product J~Z~CB free of the support. In most instances, a portion of the displacement linker remains attached to the support, as is shown in **Scheme 3**, and represented by A~Support. However, 'traceless' displacement linkers also may be employed, depending on the target ligation product and the terminus of the ligation product one desires to generate. Second linker C may be a variety of linkers for tailoring the ligation product after separation of the product J~Z~CB from the support, particular linkers that require, or benefit from, cleavage under stringent chemical conditions, such as strong acid or base conditions. It also will be appreciated that displaceable linker BA may be directly adjacent to or separated by a divalent spacer, even a polymer, from the second linker C. For instance, the spacer may be a divalent radical such as

an alkyl chain (e.g., C1-C18 or longer aliphatic), a substituted alkyl chain bearing one or more side chains (e.g., C1-C18 or longer substituted with alkyl or alcohol groups etc.), an alkyl chain having one or more heteroatoms (e.g., peptide residues, ethylene oxide, etc.), or combinations thereof. Thus the spacer, if present, may be linear, branched, substituted or unsubstituted. Typically the spacer, if present, will be stable and non-reactive under the conditions employed for ligation and displacement.

[0029] Once a desired chemical ligation product is formed (either an intermediate or full length product), the displaceable linker can be cleaved as described above, and the ligation product released free of the support. Separation of the released ligated product following displacement from the support may be carried out by removing the aqueous-based solution from the solid support material, e.g., centrifugation and/or extraction, phase partition etc., and the desired target ligation product further purified from the recovered solution following standard liquid separation protocols and chromatography depending on the target ligation product (e.g., normal and reverse phase liquid chromatography, including high pressure liquid chromatography, ion exchange, size exclusion, gel filtration, affinity chromatography, affinity chromatography, electrophoresis, capillary electrophoresis, etc).

[0030] As used herein, the term "chemical ligation" is intended to mean the formation of a covalent linkage between chemoselective coupling partners. Chemoselectivity is achieved by the presence of unique, mutually reactive, functional groups on each of the coupling partners that render the ligation reaction chemoselective. For example, the chemical ligation of peptides and polypeptides involves the chemoselective reaction of peptide or polypeptide segments bearing compatible unique, mutually reactive, C-terminal and N-terminal amino acid residues. Several different chemistries have been utilized for this purpose, preferred examples of which include amide-forming native chemical ligation (Dawson, *et al., Science* (1994) 266:776-779; Kent, *et al.*, WO 96/34878), extended general chemical ligation (Kent, *et al.*, WO 98/28434), oxime-forming chemical ligation (Rose, *et al., J. Amer. Chem. Soc.* (1994) 116:30-33), thioester forming ligation (Schnölzer, *et al., Science* (1992) 256:221-225), thioether forming ligation (Englebretsen, *et al., Tet. Letts.* (1995) 36(48):8871-8874), hydrazone forming ligation (Gaertner, *et al., Bioconj. Chem.* (1994) 5(4):333-338), and thiazolidine forming ligation and oxazolidine forming ligation (Zhang, *et al., Proc. Natl. Acad. Sci.* (1998) 95(16):9184-9189; Tam, *et al.*, WO 95/00846) or by other methods (Yan, L.Z. and Dawson, P.E., "Synthesis of Peptides and Proteins without Cysteine Residues by Native Chemical Ligation Combined with Desulfurization," *J. Am. Chem. Soc.* (2001)

123:526-533, herein incorporated by reference; Gieselnan *et al., Org. Lett.* (2001) 3(9):1331-1334; Saxon, E. *et al.*, "'Traceless' Staudinger Ligation for the Chemoselective Synthesis of Amide Bonds," *Org. Lett.* (2000) 2:2141-2143). The same basic chemistries have been applied to polymers other than peptides and polypeptides, and thus can be used to chemically ligate a wide range of polymers in accordance with the present invention.

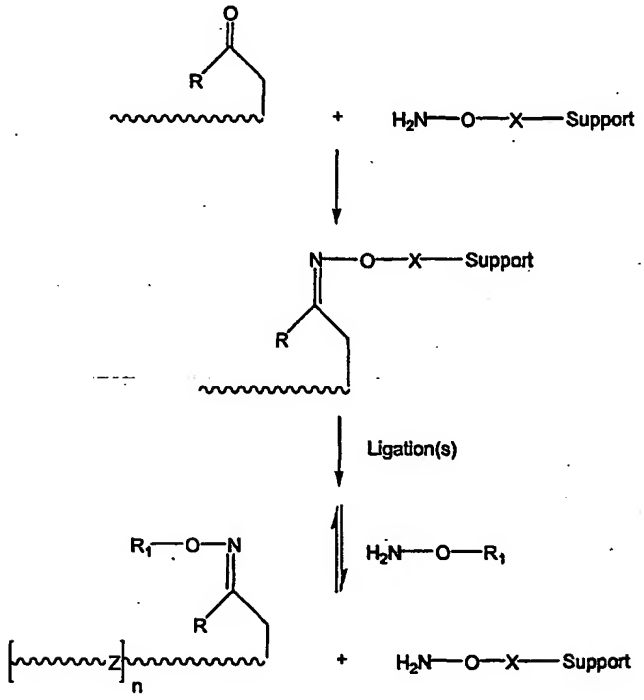
[0031] By "displaceable linker" is intended a linker capable of being cleaved or dislodged under mild aqueous conditions. By "mild aqueous conditions" is intended an aqueous or mixed organic-aqueous solution having a pH range from about 3 to 10. Such mild aqueous conditions may include excipients, such as buffer, chaotropes, detergents, lipids, salts, reducing agents, catalysts, scavengers, redox coupling agents, and the like. Linkers cleavable under mild aqueous conditions work well for coupling or linking a polymer for chemical ligation to pliable supports that swell in water, such as cellulose-, agarose- or polyethylene glycol-based supports. Thus, displaceable linkers are particularly suitable for mild cleavage and release of a ligation product from the support, thereby improving separation, purification and recovery of the desired product in general.

[0032] Displaceable linkers employable in the invention include chemical and/or enzymatic linkers. Chemically displaceable linkers are cleavable by chemical reagents or light. Preferred examples of chemically displaceable linkers include oxime, hydrazone, diol, photolabile, allyl, and metal chelator linkers. Enzymatically displaceable linkers are cleavable by an enzymatic reaction. Preferred examples of enzymatically displaceable linkers include carbohydrate linkers containing endoglycosidase recognition sequence(s), peptide linker sequences containing an endoprotease recognition sites, as well as nucleic acid linkers containing restriction enzyme recognition or autocatalytic site(s).

[0033] By way of example, oxime linkers are particularly useful chemically displaceable linkers in that they can be cleaved with a variety of compounds bearing an aminooxy functionality under mild aqueous conditions, typically at mildly acidic pHs around 3.5. For instance, (aminooxy)acetic acid (AOA) can be used as a displacement reagent for cleavage of many oxime bonds, and is exemplary of a weak acid having a pH in water of about 3 to 3.5. This is in contrast to strong acids such as trifluoroacetic acid (TFA) that have pH's less than 3 in water, which is a typical acid employed for cleavage of many other linkers, for example, a second linker employable with in the methods and compositions of the invention. Chemical linkers such as oxime linkers provide additional flexibility in that they can be provided in

different orientations relative to the polymer and support. Depending on the orientation, a ligated polymer product released from the support will bear either an aminooxy functionality or an oxime moiety, and can be exploited for a variety of post-ligation and cleavage purposes. Use of an oxime linker for the solid phase chemical ligation method and compositions of the invention are illustrated in Scheme 4 and Scheme 5.

Scheme 4

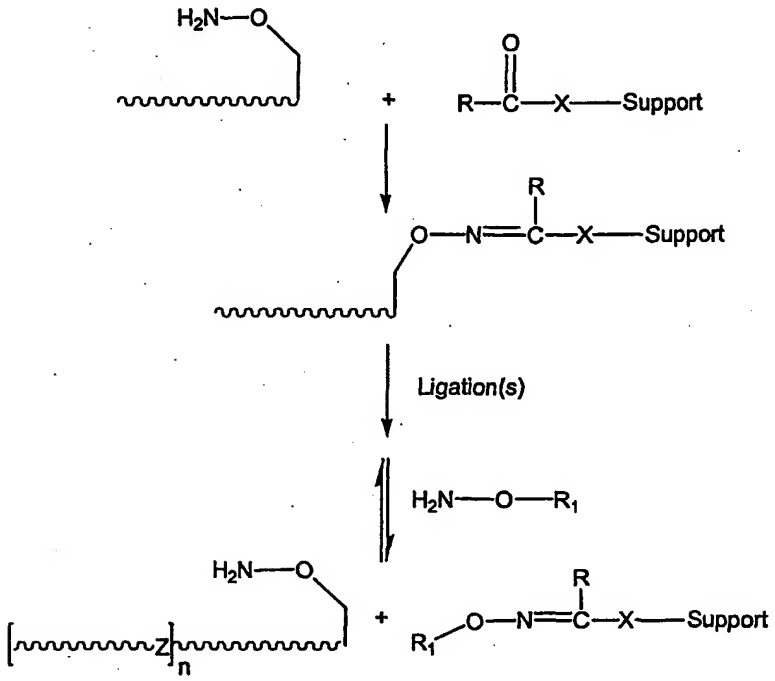


[0034] As shown in Scheme 4, the support and first polymer are constructed to bear complementary reactive groups capable of oxime formation. The first polymer is constructed to bear an aldehyde or ketone moiety (including glyoxyls), whereas the support bears an aminooxy group. Coupling of the support and the polymer forms an oxime-linked support bound first polymer. The polymer can then be subjected to one or more ligation reactions to extend or otherwise elaborate the target ligation product. Once the ligation(s) are completed, the support-bound ligation product is release from the support by the addition of an aminooxy compound, such as aminooxyacetic acid. Addition of the aminooxy compound displaces the linker, and yields a ligation product free of the support bearing an oxime moiety, with concomitant regeneration of the aminooxy on the support. In **Scheme 4**, R is any group compatible with aldehydes or ketones. Preferred examples of R include H, carbonyl, or a variety of alkyl and aryl groups. For example, R may include $-CH_3$, $-CH_2-CH_3$, $-C(O)H$, and $-C(O)-CH_3$. Substituents R_1 and X are any groups compatible with aminooxy functionalities,

and may be the same or different. Preferred examples of X and R_1 include alkyl and aryl moieties, with alkyl groups being more preferred, and may be linear, branched, substituted and unsubstituted. By way of example, an oxime linker may be formed as the reaction product of various aminooxy compounds with aldehyde or ketone acids; examples include oxime reaction products of an aminooxyacetyl compound with an aldehyde or ketone acid such as formyl, glyoxyl, pyruvic, levulinic, acetoacetic, acetonedicarboxylic acid and the like.

[0035] - As described above, the oxime linker can be provided in either orientation, depending on the intended end use. For example, in contrast to **Scheme 4**, the following **Scheme 5** depicts the situation where the polymer bears the aminooxy functionality.

Scheme 5

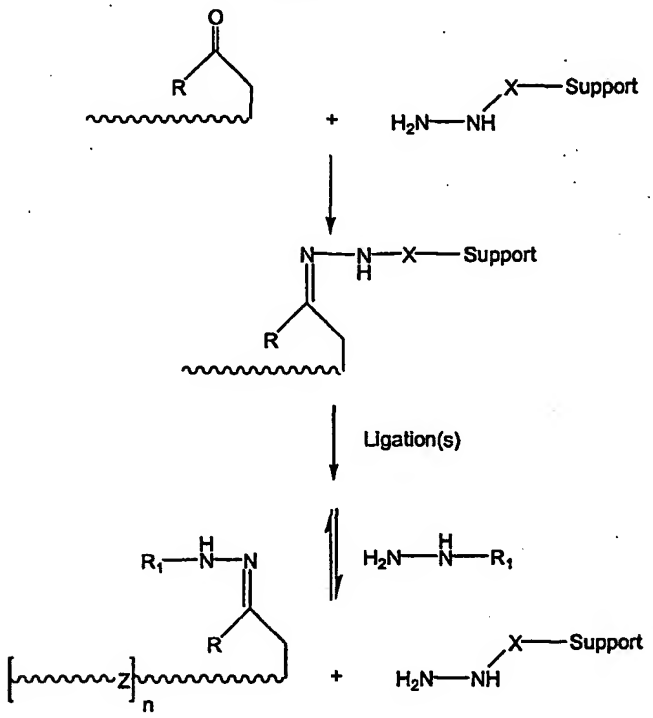


The ability to provide the displaceable bond in different orientations can be advantageous where post-ligation modifications include attachment of aldehyde or ketone bearing target molecules, such as polymers, dyes, tracers, drugs, and the like (See, e.g., EP 0 243 929 B1; WO 94/25071; US 6,001,364; US 6,174,530; and US 6,217,873; and US 6,228,654). In addition, when the polymer is designed to bear the aminooxy functionality, the residual aminooxy component following displacement from the support can be employed as a purification handle following ligations and release from the support. It also is possible to reduce the oxime for capping or stabilization purposes, as with other Schiff-base type compounds. (See, e.g., EP 0 243 929 B1; WO 94/25071; US 6,001,364; US 6,174,530; and

US 6,217,873). Although attachment of the aldehyde or ketone moiety to the first polymer can be accomplished through a pendant terminal group of the polymer, as shown in **Scheme 4**, the aldehyde or ketone moiety can be readily attached to a side chain group, if present, on a polymer. For instance, while **Scheme 4** and other reaction schemes depicted herein illustrate attachment of such groups through or to a pendant terminal group on a polymer, a displaceable linker, linker system or component thereof may be attached to a side chain group on a polymer.

[0036] Displaceable hydrazone linkers represent another preferred chemically displaceable linker of the invention. Hydrazone linkers are dislodged or otherwise cleavable by hydrazine compounds "H_2N-NH-R_1" under mild conditions, and find use in the methods and compositions of the invention as illustrated in **Scheme 6**.

Scheme 6

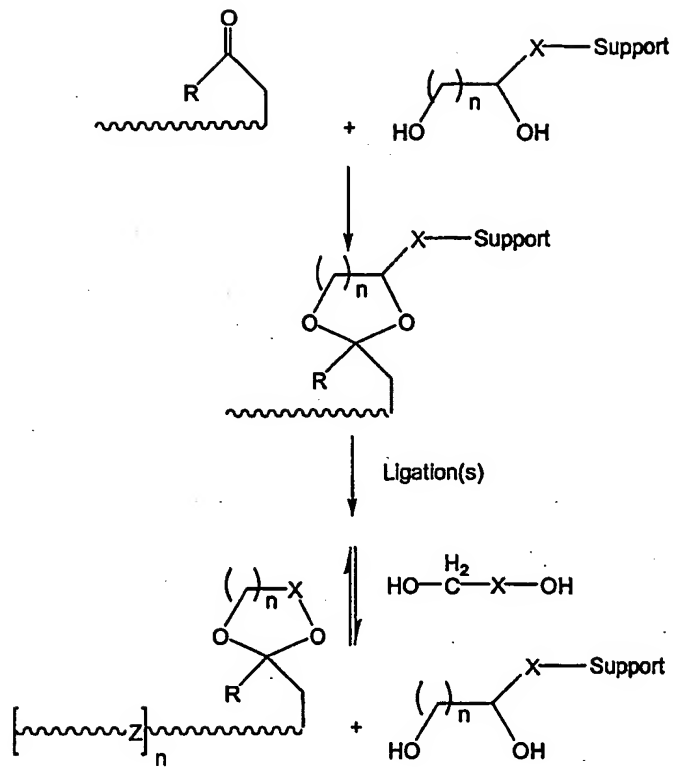


[0037] As shown in **Scheme 6**, a hydrazine-functionalized support "H_2N-NH-X~Support" is provided and reacted with an aldehyde- or ketone-functionalized first polymer "Polymer~C(O)R" to form a hydrazone-linked support-bound polymer "Polymer~C(R)-N=NH-X~Support". Following one or more ligation reactions that extend or otherwise elaborate the initial support-bound polymer, the support-bound ligation product J-[Polymer-Z]$_n$-Polymer~C(R)-N=NH-X~Support (shown as "Ligation(s)" in **Scheme 6**), where "n"

represents the number of ligated segments / reactions on support, and J is as defined above and may be present or absent, is contacted with a hydrazine compound "H_2N-NH-X-R_1." Addition of the hydrazine compound results in displacement of the hydrazone bond, and release of a ligation product bearing a hydrazide moiety from the support along with concomitant regeneration of the hydrazine-functionalized support. As with the oxime linkers, the hydrazone linkers may be formed with the hydrazide and aldehyde or ketone functionalities in different orientations (only one orientation shown in **Scheme 6**). Thus, the hydrazide functionality can be provided by the support or by the first polymer for attachment to the support. In **Scheme 6**, R is any group compatible with aldehydes or ketones. Preferred examples of R include H, carbonyl, or a variety of alkyl and aryl groups. For example, R may include -CH_3, -CH_2-CH_3, -C(O)H, and -C(O)-CH_3. Substituents R_1 and X are any groups compatible with hydrazine / hydrazide functionalities, and may be the same or different. Preferred examples of X and R_1 include alkyl and aryl moieties, and may be linear, branched, substituted and unsubstituted. Where X is an aromatic group, the resulting hydrazone bond is typically more stable, compared to where X bears a carbonyl group adjacent to the =N-NH- bond, which is more labile. By way of example, a hydrazone linker may be formed as the reaction product of various hydrazine compounds with aldehyde or ketone acids; examples include hydrazone reaction products of an hydrazide compound with an aldehyde or ketone acid such as formyl, glyoxyl, pyruvic, levulinic, acetoacetic, acetonedicarboxylic acid and the like. As with oximes and other Schiff-base type bonds, it is possible to reduce the hydrazone linkage or resulting hydrazide for capping or stabilization purposes. (See, e.g., EP 0 243 929 B1; WO 94/25071; US 6,001,364; US 6,174,530; and US 6,217,873).

[0038] In another preferred embodiment, diol linkers are employed as displaceable linkers in the methods and compositions of the invention, as illustrated in **Scheme 7**. The diol linkers are readily displaceable under mild aqueous conditions, for example, under neutral to slightly basic conditions (e.g., pH 6 to 9), and thus conditions compatible with a wide variety of supports that swell in water, such as cellulose, agarose or PEGA type resins.

Scheme 7

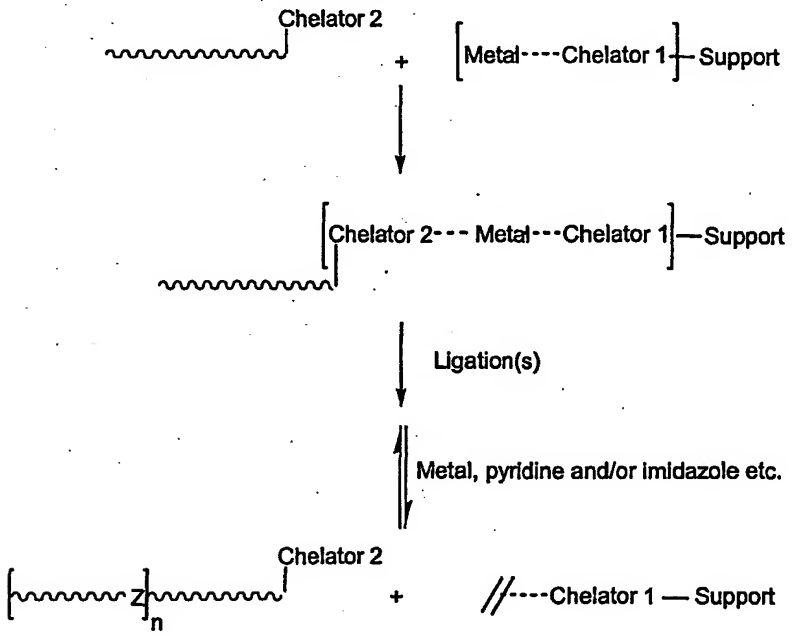


[0039] As with the oxime and hydrazone linkers, the R group of the aldehyde or ketone bearing functionality is as described above, which is shown as part of the polymer in Scheme 7, and thus is any group compatible with aldehyde or ketone groups. The group X is any group compatible with diols, and includes alkyl and aryl groups, including linear, branched, substituted and unsubstituted compounds capable of forming a suitable stable linkage with the support, where "n" is from 0 to 2. Preferred diols for coupling to the support and/or as cyclic diol displacement reagents are those capable of forming a displaceable cyclic diether group linkage (i.e., a cyclic diol) with the aldehyde or ketone moiety. Thus, preferred diols are those compounds of the formula 1,*n*-diol, i.e., having two hydroxy groups present on different carbon atoms, where the hydroxy groups are usually but not necessarily adjacent. Diols may also be displaced by mild periodate oxidation. For example, preferred diols suitable as diol-formers and/or as displacement reagents for use in the methods and compositions of the invention include compounds such as $HOCH_2CH_2OH$ 'ethylene glycol' (ethane-1,2-diol), $HO[CH_2]_3OH$ (1,3-propanediol), $HO[CH_2]_4OH$ butane-1,4-diol, $HOCH_2CH(OH)CH_3$ (1,2-propanediol propylene glycol). Diols suitable for coupling to the first polymer or support will bear a functional group in addition to the two hydroxy groups of the diol. These include halogen-functionalized 1,*n*-diols such as 3-halogenpropane-1,2-diol,

meso-1,2-diols, as well as higher alcohols, such as 1,2,3-propanetriol (glycerol, glycerin), that can be attached to a support through various techniques known in the art. Vicinal 1,2-diols, including functionalized pinacols such as tetra(hydrocarbyl)ethane-1,2-diols (e.g., $R_2C(OH)C(OH)R_2$) of which the tetramethyl example is the simplest one and is itself commonly known as pinacol (benzpinacol is the tetraphenyl analog), and 3-chloropropane-1,2-diol are preferred examples of diols suitable for attachment to a support. The diols may be provided as a substituent(s) on aryl compounds as well. The diol compounds may be purchased from commercial sources, as well as prepared de novo by numerous methods (See, e.g., Albany Molecular Research, Inc., (2002) Technical Report: Volume 6, No. 30).

[0040] Metal chelator linkers represent an additional type of a preferred chemically displaceable linker suitable for utilization in the methods and compositions of the invention. The use of metal chelator linkers in the context of the methods and compositions of the invention are illustrated in **Scheme 8**.

Scheme 8



As shown in **Scheme 8**, the support "[Metal----Chelator1]~Support" bears a metal chelate forming group "[Metal----Chelator1]" that is charged with a metal ion of interest, such as copper, zinc, cobalt, iron, nickel etc., depending on the type of metal chelating ligand immobilized on the support. The first polymer bears a metal chelator "Chelator 2" capable of binding to the metal-charged complex "[Metal----Chelator1]~Support." Following ligation of

one or more polymers, the support-bound ligation product "J-[Polymer-Z]$_n$-Polymer~[Chelator2---Metal---Chelator1]~Support" is released from the support by addition of a competing metal, salts, pH gradients, or more preferably, ligands that can displace the coordinating metals employed for charging the initial metal coordination complex, such as pyridine and/or imidazole.

[0041] This aspect of the invention may employ a variety of metal chelator linkage systems, particular those utilizable for "immobilized metal chelate affinity chromatography" (IMAC). For instance, IMAC employs free coordination sites of metal ions to bind a support and target molecule together by forming a compatible metal chelator complex therein between (See, e.g., Porath, *et al.*, *Nature* (1975) 258:598-599; US 4,897,467; US 5,141,966; US 5,185,313; EP 0 593 417 B1; and EP 0 437 875 B1). The principle IMAC mechanism, as applied to the solid phase chemical ligation methods and compositions of the invention, is based on the interaction between a metal ion coordinated to (i) an aqueous compatible support bearing a covalently bound first chelating ligand and (ii) a second chelating ligand covalently bound to a target polymer of interest that also bears a chemoselective group capable of chemical ligation. Thus, a target polymer capable of chemical ligation is bound to the support through the coordination bonds of a metal ion. The metal chelator linkage systems employed in the methods and compositions of the invention are substantially stable to conditions employed for chemical ligation. In addition, the metal chelator linkage and conditions employed for subsequent chemical ligation reactions can be selected for optimal compatibility by standard manipulation of conditions for ligation, such as described below. Following chemical ligation, displacement of the metal coordination bonds, and thus the metal ion linkage releases the desired ligation product from the support.

[0042] By way of example, iminodiacetic acid (IDA) is a suitable metal chelating ligand that forms a bidentate chelating moiety after immobilization to a support, or incorporation as a metal chelating ligand of the first polymer. The free bidentate chelating moiety can then be exploited for binding to a suitable a coordinating metal ion such as copper or nickel. For IDA, binding of the metal ion occurs through the nitrogen atom and two carboxylate oxygens, where the metal will coordinate 4-6 ligands; the remaining coordination sites are occupied by water that can be displaced by another metal chelator ligand. Thus, when IDA is immobilized on a support and charged with nickel, a target polymer bearing a group capable of chemical ligation and a group comprising a second metal chelator ligand can be attached to the Ni-IDA-Support through the remaining, free nickel coordination sites.

[0043] As noted above, the orientation of the linker can be varied, for example, instead of the polymer bearing Chelator 2, the polymer can bear the metal charging component Chelator 1 for immobilization to a support bearing a metal Chelator 2. Thus, a metal chelator such as IDA can be covalently attached to the target polymer of interest, charged with a metal ion of interest, and then attached to a support bearing a second chelator. In addition to IDA, examples of preferred metal chelating ligands for attachment to the support or target polymer of interest include, but are not limited to, cyclams, penicillamine, dimercaptosuccinic acid, tartrate, thiomalic acid, crown ethers, nitrilotriacetic acid (NTA), ethylenediaminetetraacetic acid (EDTA), ethylenebis(oxyethylenenitrilo)tetraacetic acid (EGTA), 3,6-dioxaoctanedithiomide, 3,6-dioxaoctanediamide, salicylaldoxime, dithio-oxamide, 8-hydroxyquinoline, cupferron, 2,2'-thiobis(ethyl acetoacetate), and 2,2'-dipyridyl.

[0044] For immobilizing a target polymer capable of chemical ligation, and for performing subsequent ligation reactions, it is preferred to employ tri-, tetra-, penta- and multi-dentate metal coordination ligands, as the higher multi-dentate complexes typically exhibiting greater metal affinity and stability. Thus, a diverse range of metal chelator linker systems can be employed as a displaceable metal chelator linker system of the invention. For example, in addition to the above metal chelator displaceable linkers, a variety of metal coordinating compounds, including nitrophilic, amino, carboxylates, as well as compounds with pyridine or imidazole cores (See, e.g., US 5,185,313 and EP 0 593 417 B1), polyhistidine-tagged ("his-tag") metal chelator systems (See, e.g., US 4,877,830; US 5,047,513), polymer or lipid-based metal chelators incorporating fluorophoric sensors (See, e.g., US 5,059,654; US 5,616,790), as well as suitable metal-chelating polyamines and polyamidoamides (See., e.g., US 4,332,928) can be employed. By way of example, with his-tag - nickel-chelate affinity systems, interaction between Ni^{2+} and support such as a NTA functionalized cellulose, agarose or PEGA support is very strong, and chemically resilient. NTA is an aminotricarboxylic acid that generally binds bivalent metal ions in a ratio of 1:1. So for example, when nickel is used, every Ni^{2+} ion binds to two histidine molecules in a non-conformation dependent manner, therefore it resists strong denaturants such as 6M guanidinium chloride. In addition to His-tag systems, polyamine metal chelators can be employed, and include acyclic polyamines (e.g., diethylamine, diethyltriamine, and diethyltetraamine) and macrocyclic polyamines (e.g., cyclams and cylems). Desferrioxamine and its metal chelating derivatives is another metal chelating ligand of interest. Desferrioxamine has multiple carbonyl and hydroxyl groups that provide electrons to

coordinate with those in Fe^{3+}, chelates iron in a one-to-one ratio, and has a primary amine for functional attachment to a variety of target molecules and/or supports. Desferrioxamines also can bind other "hard" ions, such as Al(III), Zn(II), Ga(III), Cr(III), and, Pu(III,IV) (hard metal ions have high charge to ionic radius ratios and form strong inner sphere complexes with ligands containing "hard" donor atoms, such as oxygen). Polypyridylalanine metal chelator linkers are another example, where a series of pyridylalanine residues can serve as a versatile metal affinity tag that can be used with many different immobilized metal ions. The occurrence of the pyridyl side chain as a ligand in coordination complexes is well documented – pyridine and its derivatives bind to a wide range of transition elements with high affinities. Pyridylalanine and related compounds (for example, homo-pyridylalanine) are available in three different isomeric forms (2-,3- and 4-pyridylalanine) as Boc or Fmoc protected derivatives ready for use in solid phase peptide synthesis (RSP Amino Acid Analogs, Inc.,; Bachem, Inc.). Although less preferred, polythiol linkers also may be employed, for instance, as a series of amino acids with thiol side chains in conjunction with low valent late transition elements (also known as "soft" metals). In addition to cysteine, other amino acids with thiol-containing side chains (homocysteine for example) are commercially available in forms suitable for use in solid phase synthesis, and utilization in solid phase chemical ligation methods and compositions of the invention.

[0045] Multi-dentate ligands can have extremely high metal affinities, and thus represent preferred metal chelator displaceable linkers of the invention. For instance, attachment of the normally hexadentate ligand EDTA to a target polymer such as a peptide through one of its carboxylate "arms" will result in a pentavalent ligand system that will still exhibit considerable binding affinity to a wide variety of metal ions. The sixth coordination site will be open to binding to ligands immobilized onto solid supports, such as pyridine, thiol or imidazole modified aminomethyl supports. These supports may be prepared by simple reaction between halogenated derivatives of these ligands with the free base.

[0046] In a preferred embodiment, a target first polymer of interest is tagged with a string of metal-chelating amino acid residues at a terminal end opposite of the terminal end of the polymer bearing the chemoselective ligation group. For instance, when the target polymer is a peptide or polypeptide, a string of histidines can be readily incorporated at the N- or C-terminus. A sufficient number of histidine residues are provided in such a string so as to be capable of forming an electron rich metal chelator. For instance, four to six histidine residues in sequence are sufficient for this purpose. Other sequences, such as Serine-Proline-Glycine-

Histidine-Histidine-Glycine, can also be employed to vary metal preference and elution parameters, as well as Histidine-Tryptophan sequences. (Smith, *et al., J. Biol. Chem.* (1988) 263:7211-7215).

[0047] In general, however, the affinity of a suitable four to six long "His-tag" for Cu^{2+}, Ni^{2+}, Co^{2+} or Zn^{2+} allows a His-tagged polymer to be efficiently bound to a metal chelate affinity support, such as a nickel-agarose or a special metal-charged resin column (See, e.g., Hochuli *et al., Bio/Technology* (1988) 6:1321-1325). Following ligations, the ligation product of interest that is bound to the matrix through interactions with the metal ions and can be eluted with a solution of ammonium chloride, glycine, histidine, imidizole, EDTA or high levels of competing metal ions (See, e.g., Kagedal, L. in Protein Purification: Principles, High Resolution Methods, and Applications (Janson, J.C. and Ryden, L., Eds.) (1989) pp. 227-251, VCH). A number of companies make suitable metal chelate supports for his-tagged or other chelator-tagged polymers of interest, e.g., Novagen's HIS-BIND nickel resin (Novagen, Inc., Madison, WI, USA); Clonetech's TALON cobalt resins (Clonetech, Inc, Palo Alto, CA, USA); as well as Qiagen's Ni-NTA (nickel-nitriloacetic acid) spin columns (Qiagen Inc., Chatsworth, CA, USA).

[0048] When a displaceable metal chelator linker system is employed, excipients, if any, are chosen so at not to significantly alter the redox state of the metal, or remove the metal completely, used for immobilizing the first polymer to the support, and in performing subsequent ligations until release of the product from the support is desired. For example, when using Ni-NTA supports, care should be taken to limit reducing agents such as DTT, chelating agents such as EDTA and EGTA above 1 mM and certain buffers such as Tris, HEPES and MOPS, which contain secondary or tertiary amines; however, the following provides an exemplary list of preferred excipients that can be employed with Ni-NTA supports, as well as their associated higher end concentrations: 6 M guanidinium chloride, 50% glycerol, 8 M urea, 20% ethanol, 2% Triton X-100, 2 M NaCl, 2% Tween 20, 4 M $MgCl_2$, 1% CHAPS, 5 mM $CaCl_2$, 20 mM β-mercaptoethanol, and $\leq$20 mM imidazole. These parameters are used as a guide, but can be altered and/or optimized depending on the intended end use. For instance, higher concentrations of β-mercaptoethanol (e.g., 20% of solution) can be used in some cases, such as after Acm removal to wash away excess mercury for Ni-NTA / His-tag systems. The same principles are applied for other metal chelator linkers systems. It will be appreciated that the above conditions can be varied or determined de novo for any particular condition and/or excipient, and thus the range and optimal

conditions employed can be determined for any particular displaceable metal chelator linker system and ligation scheme by one of ordinary skill in the art.

[0049] Another consideration when a displaceable metal chelator linker system is employed is the pH of buffers and/or other solutions or conditions employed prior to a desired cleavage event of a ligation product from the support. In particular, it is preferred to employ a pH that avoids lowering the metal affinity of a system prior to a desired cleavage event, including conditions employed for removal of any protecting groups during and/or after ligations. The range and optimal pH conditions can be determined for any particular displaceable metal chelator linker system and ligation scheme by one of ordinary skill in the art. In general, the pH of a solution for solid phase ligations with a displaceable metal chelator linker system of the invention will be above pH 4, with a pH of around or above 4.5, 5.0, 5.5, and with a pH of 6.0 and above being most preferred. It also will be appreciated that while the reactions can be employed at higher pH's without significant unwanted cleavage, it is preferable to employ solutions that have a pH around or less than 10, with a pH around or below 9.5, 8.0 and 7.5 or below being most preferred. Again, the range and optimal pH conditions for any particular reaction and metal chelator linker system employed can be readily determined by one of ordinary skill in the art. By way of example, when ligations involving a peptide polymer bearing a His-tag that is bound to a support through Ni-NTA system, the pH of buffers or other solutions should typically be above pH 6, and preferably between pH 6 and 7.5. This pH range is sufficient for maintaining metal affinity and binding, as well as, for example, performing many chemical ligation reactions or other manipulations, such as removal of an Acm protecting group from the N-terminal cysteine of a Ni-NTA support-bound His-tag peptide for a subsequent round of on-support ligation with an incoming thioester or selenoester bearing peptide. In contrast, by lowering the pH, such as with typical Acm removal conditions in the presence of sodium acetate at pH 4.0, the polymer may be released from the Ni-NTA support.

[0050] Additional examples of chemically displaceable linkers include photolabile linkers. Preferred examples are supports functionalized with 3-hydroxymethyl-4-nitrophenoxymethyl (Nicolaou, *et al.*, *Angew. Chem.* (1998) 110:1636), or safety-catch type photolabile linkers such as 3-(hydroxy(2-phenyl-1,3-dithian-2-yl)methyl)phenoxymethyl supports (Routledge, *et al.*, *Tet. Lett.* (1997) 38:1227). Additional examples include photolabile pivaloylglycol anchor / linker groups (Peukert, *et al.*, *J. Org. Chem.* (1998) 63(24):9045-9051). A preferred photolabile linker is the acid- and base-stable linker 3-amino-3-(2'nitrophenyl)-2,2-

dimethylpropionic acid (Sternson, *et al.*, Tet. Lett. (1998) 39:7451-7454). Photolabile linkers have the advantage of being employed under a variety of chemical conditions for support-bound polymer ligations, followed by release of the ligation product via irradiation, typically by ultraviolet irradiation. However, care is taken to minimize exposure of the support and ligation products to any light until cleavage is desired.

[0051] Reducible linkers are those that are cleavable in the presence of a reducing agent or otherwise under reducing conditions. For example, allylic linkers, such as HYCRAM (hydroxy-crotonyl-aminomethyl; Kunz and Dombo, *Angew. Chem.* (1988) 100:733-734) and HYCRON (Seitz and Kunz, *J. Org. Chem.* (1997) 62:813-826), are examples of chemically displaceable linkers suitable for use with the compositions and methods of the invention. The allylic linkers are particularly useful in that they are stable under a variety of solid phase synthesis conditions, and can be cleaved under mild, aqueous conditions with a hydrogen-generating catalyst, e.g., a palladium catalyst in the presence of nucleophiles. Disulfide-forming linkers are additional examples of reducible linkers, which can be cleaved by reducing agents, e.g., thiols and the like.

[0052] Enzymatically displaceable linkers are cleavable by an enzymatic reaction. Many suitable enzymatically displaceable linkers are known and can be used for this purpose, as the conditions for exploiting such enzymes are typically mild, aqueous conditions (See, e.g., Waldman, *et al., Angew. Chem.* (1995) 107:2425-2428; Elmore, *et al., J. Chem. Soc., Chem. Commun.* (1992) 14:1033-1034; Schuster, *et al., J. Amer. Chem. Soc.* (1994) 116:1135-1136; US Pat. No. 5,369,017; and US Pat. No. 6,271,345). For example, US 6,271,345 discloses linkers suitable for cleavage by hydrolytic enzymes, including linkages such as amide, ether, phosphoric ester or glycoside linkages, where the hydrolytic enzymes include lipases, esterases, amidases, proteases, peptidases, phosphatases, peroxidases or glycosidases. Thus, for example, suitable enzymatically displaceable linkers include these, and in particular, for instance, carbohydrate linkers containing endoglycosidase recognition sequence(s), peptide linker sequences containing an endoprotease recognition sites, as well as nucleic acid linkers containing unique restriction enzyme recognition site(s), or autocatalytic nucleic acid sequence that can be caused to self-cleave under defined conditions. Glycosidases include enzymes capable of releasing N-linked oligomannose, such as PNGase F, Endo H, Endo D and Endo F1; carbohydrate linkers can be prepared from natural sources or made synthetically following standard protocols with a variety of coupling strategies (See, e.g., US Pat. No. 6,242,583; and Sears, *et al.*, *Science* (2001) 291:2344-2350). Nucleic acid linkers

can be displaced by competition with complementary oligonucleotides, by melting, or by restriction enzymes, ribozymes, or autocatalysis, and can be attached to a support following numerous standard protocols (Jaschke, *et al.*, *Tet. Lett.* (1993) 34:301-304; Ma, *et al.*, *Nucleic Acids Res.* (1993) 21:2585-2589; Hendry, *et al.*, *Biochemica Biophysica Acta* (1994) 1219:405-412; US Pat. Nos. 6,127,173; US 5,298,612; US 5,334,711; US 5,414,077; US 5,539,097; US 5,807,718; US 5,861,501; US 5,688,670; and US 5,688,940). Lipases and esterases are particularly useful for generating carboxyl groups. (Sauerbrei, *et al.*, *Angew Chem. Int. Ed.* (1998) 37:1143-1146).

[0053] Depending on the polymer employed for ligation, different displaceable linkers may be employed. In general, displaceable linkers are typically selected to cleave a unique site not present in the polymers subject to ligation. For instance, where a polymer employed in ligation includes a carbohydrate, then a displaceable linker other than an endoglucanase can be used. Where the polymer includes a peptide or polypeptide, then peptide linkers, containing a cleavage site not found in the polymer sequences, may be employed. For instance, the endoprotease Factor Xa cleaves after the arginine residue in its preferred cleavage site Ile-(Glu or Asp)-Gly-Arg (Nagai, *et al.*, *PNAS* (1985) 82:7252-7255; Quinlan, *et al.*, *J. Cell Sci.* (1989) 93:71-83; and Eaton, *et al.*, *Biochem.* (1986) 25:505-512). Factor Xa is particularly useful given the low occurrence of such recognition sequences in peptides and polypeptides. Other enzymes that find use in the invention will be apparent to one of ordinary skill in the art, such as thrombin and enterokinase enzymes and their corresponding protease recognition sequences.

[0054] As noted above, a preferred linker system of the invention comprises a displaceable linker and a second linker that is cleavable under conditions orthogonal to displacement of the displaceable linker, where the second linker joins the polymer to the displaceable linker, and the displaceable linker is attached to the support. In general, the second linker is chosen to be substantially stable to the conditions employed for chemical ligation. This is particularly true for multi-ligation strategies carried out on the solid support. And as the second linker can be cleaved after the ligation product is separated from the support by cleavage of the displaceable linker, the second linker can be one that is cleaved under mild or even relatively harsh chemical or physical conditions that could otherwise damage the support. Thus, the second linker can be chosen to be suitably stable under aqueous conditions for carrying out the chemical ligation reaction(s), but cleavable under more stringent chemical or physical conditions.

[0055] Accordingly, a wide range of second linkers may be employed in the methods and compositions of the invention. Suitable second linkers include, for example, PAL, XAL, PAM, RINK, SCAL and Sieber-based linker systems (e.g., PAL (5-(4'-aminomethyl-3',5'-dimethoxyphenoxy)valeric acid, XAL (5-(9-aminoxanthen-2-oxy)valeric acid), 4-(alpha-aminobenzyl)phenoxyacetic acid, 4-(alpha-amino-4'-methoxybenzyl)phenoxybutyric acid, p-alkoxybenzyl (PAB) linkers, photolabile o-nitrobenzyl ester linkers, 4-(alpha-amino-4'-methoxybenzyl)-2-methylphenoxyacetic acid, 2-hydroxyethylsulfonylacetic acid, 2-(4-carboxyphenylsulfonyl)ethanol, (5-(4'-aminomethyl-3',5'-dimethoxyphenoxy)valeric acid) linkers, WANG hydroxymethyl phenoxy-based linkers, RINK trialkoxybenzydrol and trialkoxybenzhydramine linkers, PAM phenylacetamidomethyl, SCAL-type safety catch acid labile linkers, and Sieber aminoxanthenyl linkers). These linker systems are cleavable under well known acidolysis conditions (typically trifluoroacetic acid (TFA) or hydrogen fluoride (HF)), UV photolysis ($\lambda \approx 350$ nm) conditions, or catalytic hydrogenation conditions. In contrast, ester-forming linkers are examples of those typically cleavable in base, for example, HMBA (4-hydroxymethylbenzoic acid) and HMBA (4-methylbenzhydrylamine) linkers are stable to strong acids, including TFA, but cleavable in base; and carboxylic acids can be attached to this linker using the activator MSNT (1-(Mesitylene-2-sulfonyl)-3-nitro-1H-1,2,4-triazole) in the presence of 1-methylimidazole. It will be appreciated that derivatives of such linkers and other linker systems may also be used. The most preferred linkers are cleavable in acidic conditions or light. For instance, the physical integrity of biological polymers such as peptides, polypeptides, nucleic acid, carbohydrates and the like are better maintained under acidic conditions.

[0056] As noted above, the second linker is typically chosen depending on the type of residual group one desires to generate on the final ligation product. So for example, the following linkers can be used to generate carboxylates: PAM, DHPP (4-(1',1'-dimethyl-1'-hydropropyl)phoxyacetyl), Wang acid, SASRIN, HAL (hypersensitive Acid-Labile (HAL) tris(alkoxy)benzyl ester), Trityl, Rink acid, SAC (silyl acid), α-methylphenacyl ester, allylic linkers, and fluorene derived N-[9-hydroxymethyl-2-fluorenyl] succinamic acid (HMFS) linkers. The following are examples of linkers that can be used for generating amides: PAL, Rink amide, XAN (xanthen-9-yl), photolabile amide linkers, SCAL and oxime linkers can be used. For generating alcohols and amines, the following are example of linkers that can be used: THP (tetrahydropyranyl) and Silyl chloride linkers for alcohols, as well as ketal and acetal linkers for diols and alcohols, and p-nitrophenyl carbonate for amines, and REM for

the preparation of tertiary amines. Many additional linkers are known, and their preparation and use are replete in the literature, and can be used to generate the desired group.

[0057] Of course, the second linker may be displaceable as well. To avoid redundancy, when two displaceable linkers are employed, with one being the second linker, the displaceable linkers should be orthogonal. For instance, a displaceable oxime linker may be employed in combination with a displaceable metal chelator linker as so on. Thus, orthogonal linker schemes may be employed where two or more classes or groups are cleaved by differing chemical mechanisms, and therefore can be removed in any order and in the presence of the other classes. Orthogonal schemes offer the possibility of substantially milder overall conditions, because selectivity can be attained on the basis of differences in chemistry rather than reaction rates. Such orthogonal approaches are applicable when choosing a particular linker and chemical ligation system.

[0058] With respect to the support, as noted above, preferred supports are those that are capable of swelling in water. These include cellulose, dextran, agarose, PEGA, or other biocompatible supports. Of course, supports that do not swell in water may also be employed, provided they are not irreversibly collapsed in water or aqueous-based solutions. For instance, controlled glass pore (CGP) supports can be used, as they are not irreversibly collapsed in water or aqueous-based solutions. In certain embodiments, CPG supports can be provided with a non-ionic hydrophilic coating, which is compatible with aqueous and most other solvent systems. The coating eliminates or reduces non-specific adsorption experienced with uncoated-CPG. In other examples, typical non-aqueous swelling supports can be modified with water-soluble polymers so that they combine both the features of a non-swelling and swelling support. For instance, resins that provide a more polar environment than polystyrene can be helpful for reducing aggregation of polymers with this tendency, such as peptides. TentaGel, reACTagel, PEG crosslinked, co-PEG, and pyridinyl resins are some examples. TentaGel and reACTagel resins incorporate polyethylene glycol chains between the polystyrene bead and the linker. Co-PEG resins are similar to corresponding polystyrene resins, but have polyethylene chains grafted to the polystyrene to provide a more polar environment. PEG crosslinked resins are polystyrene crosslinked with polyethylene glycol, which not only produces a more polar environment but also creates larger pores in the swollen support. The pyridyl resins incorporate pyridine groups into the resin that help to keep the attached polymers in a non-aggregated state.

[0059] The supports may be constructed de novo or purchased commercially. Typically, the supports are prepared or otherwise provided with an initial functional group for elaboration with the displaceable linker (or linker component thereof, such as aminooxy group for forming an displaceable oxime linker) following standard coupling procedures. For instance, oxime-forming and hydrazone-forming supports can be readily prepared from amine-functionalized supports. The amine is then modified to bear an aldehyde or ketone, or an aminooxy or hydrazide following standard procedures. Protocols for attaching these and other displaceable linker components are well known.

[0060] For instance, amino-functionalized supports are readily available from numerous vendors, and provide a universal group for immobilization of an initial displaceable linker component. The amino group can be coupled under a variety of conditions to yield a variety of stable covalent bonds. Various amino groups may be employed for this purpose, including primary amine-functionalized supports, as well as aminoaryl-supports. The later have an arylamine that can be converted to diazonium, which is capable of reacting with imidazoles and phenolic compounds, and thus useful when a diazo linkage is required for linker immobilization.

[0061] Where the incoming displaceable linker component bears an N-nucleophile such as an amino group for attachment to the support, carboxy-activated supports can be used. The carboxyl group can be activated with carbodiimide to form a pseudourea, which reacts with an amine to form an amide bond. The carboxyl-support can also be converted to N-hydroxysuccinimide by reacting with N-hydroxysuccinimide (NHS) in the presence of carbodiimide. This activated support readily reacts with ligands with free amines under very mild conditions. More generally, carbodiimide (CDI)-activated supports can be prepared or purchased and used where the immobilization occurs through the reaction of an N-nucleophile with the imidazolyl carbamate of the support to form a stable, uncharged N-alkylcarbamate linkage. Halogen-functionalized supports may also be used for coupling to nucleophile-bearing linker components.

[0062] Glyceryl-functionalized supports have an adjacent diol group on the two terminal carbon atoms, which permits the formation of displaceable diol linkages, or oxidation to an active aldehyde by meta-sodium periodate, and thus useful for constructing displaceable oxime-forming or hydrazone-forming linkages, or for attachment of an initial displaceable linker component through an amine through reductive amination.

[0063] Hydrazide-functionalized supports will typically include an aliphatic arm terminating in an active hydrazide group, which can react readily with ketone or aldehyde groups to form a hydrazone bond. The hydrazide supports are particularly suitable for coupling carbohydrates that have been modified to bear aldehyde or ketone groups (e.g., through mild periodate oxidation, which allows the cis-diols of the sugars to be transformed into reactive aldehyde moieties; these aldehydes then combine with hydrazide groups on the matrix to form leak-resistant linkages).

[0064] Epoxide chemistry is another useful way to immobilize a displaceable linker component bearing a nucleophile for attachment to the support such as an amino, thiol or hydroxy (including phenolic) functional groups. Expoxide-activated supports can be produced by the immobilization of bifunctional oxiranes such as 1,4-butanediol diglycidyl ether onto agarose or other supports. These activated supports have limited stability in aqueous medium, so once activated are typically used immediately to couple the initial displaceable linker component.

[0065] To immobilize displaceable linker components that contain no easily reactive functional groups, active hydrogens can be exploited in a Mannich reaction in which the formaldehyde and an amine are condensed in the Mannich reaction. Hydrogens in ketones, esters, phenols, acetylenes, α-picolines, quinaldines and other compounds may be aminoalkylated using the Mannich reaction. This is approach is useful for immobilizing linkers that contain no available "handles" for easy immobilization, or that have functional groups with low reactivity or that are sterically hindered. Moreover, the Mannich reaction provides an alternative to the diazonium coupling method.

[0066] Thiol-functionalized supports bear active thiol groups and can be used for immobilization through disulfide or thioether bonds. Thiol-functionalized supports are particularly useful for coupling halogen- or maleimide-functionalized linkers. Of course many other types of functionalized supports can be employed, including those formed by various cross-linking agents, such as ethylene glycolbis (succinimidylsuccinate) (EGS), which creates a hydroxylamine-sensitive linkage; bis[2-succinimidooxycarbonyloxy)ethyl]sulfone (BSOCOES), which gives a base-sensitive sulfone linkage; disuccinimidyl tartarate (DST), which introduces 1,2-diols cleavable by periodate; and dithiobis(succinimidylpropionate)(DSP), which results in thiol-cleavable or reducible disulfide bonds.

[0067] The supports may include spacer arms and the like, and when purchased commercially these options are typically available. For instance, the functional group of a support can be attached through short or longer extension arms. Longer spacer arms may help to facilitate the attachment and the approach of bulky molecules, where steric hindrance and surface repulsion can pose problems. Suitable spacer arms include aliphatic or water-soluble polymers, such as polyethylene glycol and the like.

[0068] The above and other suitable solid supports, as well as preferred linkers, their preparation and suitable conditions and protocols for their application are described in, for example, "Advanced Chemtech Handbook of Combinatorial & Solid Phase Organic Chemistry," W.D. Bennet, J.W. Christensen, L.K. Hamaker, M.L. Peterson, M.R. Rhodes, and H.H. Saneii, Eds., Advanced Chemtech, 1998, and elsewhere (See, e.g., G.B. Fields, *et al.*, Synthetic Peptides: A User's Guide, 1990, 77-183, G.A. Grant, Ed., W.H. Freeman and Co., New York; NovaBiochem Catalog 2000; "Synthetic Peptides, A User's Guide," G.A. Grant, Ed., W.H. Freeman & Company, New York, NY, 1992; "Principles of Peptide Synthesis, 2nd ed.," M. Bodanszky, Ed., Springer-Verlag, 1993; "The Practice of Peptide Synthesis, 2nd ed.," M. Bodanszky and A. Bodanszky, Eds., Springer-Verlag, 1994; "Fmoc Solid Phase Peptide Synthesis, A Practical Approach", W.C. Chan and P.D. White, Eds., Oxford Press 2000; and Bioconjugate Techniques, Greg T. Hermanson, Academic Press, 1996. (Available from Pierce as Product # 20002)). Commercial sources for functionalized supports and linkers systems include, for example, Sigma-Aldrich, Pierce Biotechnology, NovaBiochem, Advanced Chemtech, InnovaChem, CPG-Inc, Amersham Biosciences, Qiagen, ISCOS, RAPP Polymere, Agarose Bead Technologies, and the like.

[0069] As noted above, the solid phase chemical ligation method of the present invention can be employed with any aqueous-based ligation method, or combinations of ligation methods, for example, such as amide-forming native chemical ligation (Dawson, *et al.*, *Science* (1994) 266:776-779; Kent, *et al.*, WO 96/34878), extended general chemical ligation (Kent, *et al.*, WO 98/28434), oxime-forming chemical ligation (Rose, *et al.*, *J. Amer. Chem. Soc.* (1994) 116:30-33), thioester forming ligation (Schnölzer, et al., *Science* (1992) 256:221-225), thioether forming ligation (Englebretsen, *et al.*, *Tet. Letts.* (1995) 36(48):8871-8874), hydrazone forming ligation (Gaertner, *et al.*, *Bioconj. Chem.* (1994) 5(4):333-338), and thiazolidine forming ligation and oxazolidine forming ligation (Zhang, *et al.*, *Proc. Natl. Acad. Sci.* (1998) 95(16):9184-9189; Tam, *et al.*, WO 95/00846) or by other methods (Yan, L.Z. and Dawson, P.E., "Synthesis of Peptides and Proteins without Cysteine Residues by

Native Chemical Ligation Combined with Desulfurization," *J. Am. Chem. Soc.* (2001) 123:526-533, herein incorporated by reference; Gieselnan *et al.*, *Org. Lett.* (2001) 3(9):1331-1334; Saxon, E. *et al.*, "'Traceless' Staudinger Ligation for the Chemoselective Synthesis of Amide Bonds," *Org. Lett.* (2000), 2: 2141-2143).

[0070] When choosing a particular ligation chemistry, the linker system, and in particular the displaceable linker is chosen such that the ligation, linker system and conditions utilized for the combined application thereof are mutually compatible, i.e., utilize orthogonal reagents and/or conditions to perform ligation versus linker cleavage. Alternatively, safety-catch linker strategies may be employed (e.g., photolabile displaceable linker). For instance, when performing oxime forming chemical ligation, the linker system may employ a displaceable linker other than an oxime. The basic considerations for maintaining orthogonality can thus be chosen accordingly.

[0071] As is apparent, the methods and compositions of the invention have many uses. They may be used in manual and/or rapid automated synthesis schemes using conventional peptide synthesis and other organic synthesis strategies. They also are particularly useful in expanding the utility of chemical ligation to multi-component ligation schemes, such as when producing a polypeptide involving orthogonal ligation strategies, such as a three or more segment ligation scheme or convergent ligation synthesis schemes.

[0072] The methods and compositions of the invention are exceptionally useful for ligating peptides, polypeptides and other polymers. For instance, the ability to carry out solid phase chemical ligation under aqueous conditions using a variety of displaceable linkers expands the scope of chemical ligation, in general. The invention can also be used to ligate polymers in addition to peptide or polypeptide segments when it is desirable to join such moieties through a displaceable linker.The invention also finds use in the production of a wide range of peptide labels for expressed-protein ligation (EPL) applications. For instance, EPL-generated thioester polypeptides can be ligated to a wide range of peptides, depending on the intended end use, via the displaceable linker chemistries of the invention. Likewise, the invention can also be exploited to produce a variety of cyclic peptides and polypeptides

[0073] The advantages of the invention are numerous. While water-compatible polymer supports such as cellulose based materials are often used for protein-based affinity purifications, and are designed to support the immobilization of large polypeptides and proteins on their surfaces, these materials can be destroyed by the harsh conditions used for

the cleavage of standard linking systems used in peptide chemistry (e.g., anhydrous HF or TFA). This makes it difficult to recover good material in desired amounts. Accordingly, the displaceable linker strategy of the invention is particularly advantageous when water-compatible polymer supports, such as cellulose-based materials, are employed in aqueous-based solid phase chemical ligation reactions. Use of a first linker that includes a displaceable group permits facile attachment to the support, as well as removal from the support under relatively mild conditions. The permits selection and use of a second linker that is removable under relatively harsh conditions to generate the desired terminal group on the ligation product free of the support, e.g., a free carboxylate on the final ligation product following displacement of the first linker and recovery of the full-length ligation product from the support.

[0074] Also, the displaceable linker strategy employed with solid phase chemical ligation streamlines the process of obtaining chemically synthesized proteins requiring multiple ligations. By performing multiple peptide ligation and deprotection steps on polymer support, the chemical synthesis of ligation intermediates and full-length products can be achieved without the need for the isolation and purification of synthetic intermediates. The advantage of polymer supported peptide ligations over their solution counterparts will increase with the number of steps required to assemble the desired protein sequence. This technology should be readily applicable to the preparation of both small and large protein sequences requiring only a few to many ligation steps. Convenient chemical access should facilitate further engineering of longer protein sequences of interest for research or therapeutic uses, small or large scale manufacturing, rapid analoging, on-resin modification, attachment of products to protein chips and other surfaces, as well as for specific tailoring of terminal sequences. The methods and compositions also substantially increase the speed of the chemical protein synthesis approach, and permit facile recovery and improved yields due to a reduction in the need for the isolation and purification of synthetic intermediates.

[0075] All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

EXAMPLES

[0076] The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the present invention,

and are not intended to limit the scope of what the inventors regard as their invention nor are they intended to represent that the experiments below are all or the only experiments performed. Efforts have been made to ensure accuracy with respect to numbers used (e.g. amounts, temperature, etc.) but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is weight average molecular weight, temperature is in degrees Centigrade, and pressure is at or near atmospheric.

Abbreviations

Acm	acetamidomethyl
Aloc	allyoxycarbonyl
BOP	benzotriazol-1-yloxytris (dimethylamino) phosphonium hexafluorophosphate
Br,Cl Z	Br,Cl Benzylcarbamate
DCM	dichloromethane
DDE	4,4-dimethyl-2,6-dioxocycloex 1-ylidene
DIPCDI	*N,N*-diisopropylcarbodiimide
DIEA	*N,N*-diisopropylethylamine
DMAP	4-dimethylaminopyridine
DMF	*N,N*-dimethylformamide
DMSO	dimethylsulfoxide
EtOH	ethanol
Fmoc	9-fluorenylmethoxycarbonyl
FM	9-Fluorenylmethyl
HATU	(*N*-[(dimethylamino)-1H-1,2,3-triazol [4,5-b] pyridiylmethylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide).
HBTU	*N*-[(1-H-benzotriazol-1-yl)(dimethylamine)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide previously named *0*-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HF	hydrofluoric acid
HMP resin	4-hydroxymethylphenoxy resin; *p*alkoxybenzyl alcohol resin; or Wang resin
HOAt	1-hydroxy-7-azabenzotriazole
HOBt	1-hydroxybenzotriazole
Mbh	dimethoxybenzhydryl
MBHA resin	4-methylbenzhydrylamine resin

Meb	p-MethylBenzyl
MMA	N-methylmercaptoacetamide
Mmt	p-Methoxytriityl
Mob	p-MethoxyBenzyl
Msc	2-Methylsulfoethylcarbamate
Msz	4-Methylsulfinylbenzylcarbamate
Mtr	4-methoxy-2,3,6-trimethylbenzene sulfonyl
Mtt	4-methyltrityl
NMM	*N*-methylmorpholine
NMP	*N*-methylpyrrolidone, *N*-methyl-2-pyrrolidone
Nsc	4-nitrophenylethylsulfonyl-ethyloxycarbonyl
OPfp	pentafluorophenyl ester
O*t*Bu	*tert*-butyl ester
PAC	peptide acid linker
PAL	peptide amide linker
Pbf	2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl
PEG-PS	polyethylene glycol-polystyrene
Picolyl	methyl-pyridyl
Pmc	2,2,4,6,8-pentamethylchroman-6-sulfonyl
PyAOP	7-azabenzotriazol-1-1yloxtris (pyrrolidino) phosphonium hexafluorophosphate
S-*t*Bu	*tert*-butyl-thio
Tacam	Trimethylacetamidomethyl
*t*Boc	*tert*-butyloxycarbonyl
TBTU	0-(benzotriazol-1-yl)-1,1,3,3-tetramethyl uronium tetrafluoroborate
*t*Bu	*tert*-butyl
TFA	trifluoroacetic acid
Tis	Trisisopropylsilane
Tmob	2,4,6-trimehoxybenzyl
TMOF	trimethylorthoformate
Troc	2,2,2-Trichloroethylcarbamate
Trt	triphenylmethyl

EXAMPLE 1: GENERAL MATERIALS AND METHODS

Peptide Synthesis

[0077] For Boc-SPPS, peptides were synthesized in stepwise fashion either manually or on a ABI 433 peptide synthesizer by SPPS using *in situ* neutralization/HBTU activation protocols on an Boc-AA–O-CH_2-PAM resin for non-thioester peptides, or on a thioester-generating resin for thioester peptides following standard protocols (Hackeng, *et al.*, supra; Schnolzer, *et al.*, *Int. J. Pept. Prot. Res.*, (1992) 40:180-193; and Kent, S.B.H., *Ann. Rev. Biochem.* (1988) 57:957-984). Unless otherwise specified, a standard set of Boc SPPS protecting groups were used, namely: Arg(Tos); Asp(cHex); Asn(Xan); Cys(4MeBzl) and Cys(Acm); Glu(cHex); His(Bom); Lys(ClZ); Ser(Bzl); Thr(Bzl); Trp(formyl); Tyr(BrZ); Met, Gln were side-chain unprotected. After chain assembly, the peptides were deprotected and simultaneously cleaved by treatment with anhydrous hydrogen fluoride (HF) with 5% p-cresol and lyophilized and purified by preparative C4 reverse-phase-high pressure liquid chromatography (RP-HPLC).

[0078] For Fmoc-SPPS, peptides were synthesized in a stepwise manner either manually or on an ABI 433 peptide synthesizer by SPPS using HBTU/DIEA/DMF coupling protocols at 0.1 mmol equivalent resin scale. For each coupling cycle, 1 mmol N^{α}-Fmoc-amino acid, 4 mmol DIEA and 1 mmol equivalents of HBTU were used. The concentration of the activated HBTU-activated Fmoc amino acids were 0.5 M in DMF, and the couple time was 10 min. Unless otherwise specified, a standard set of Fmoc SPPS protecting groups were used, namely: Cys(Acm), Lys(Mtt), and Ser(OtBu) or Ser(OBzl). After chain assembly, the Fmoc peptides were deprotected by treatment with 20% piperidine in DMF solution for 2 x3 minutes. Deprotected peptide resin was then drained, and washed with DCM, DMF, DCM, and then dried *in vacuo* for 1 h. The peptide-resin was then cleaved by treatment with a TFA/TIS/H_2O (95:2.5:2.5) solution at room temperature for 1 h. The volatiles were then removed with a stream of nitrogen over 10 minutes and product extracted with 50% acetonitrile/water. The resin was filtered off and the aqueous solution containing the desired peptide thioester free of the resin lyophilized, followed by purification using preparative C4 RP-HPLC.

[0079] HPLC fractions containing pure peptide were identified using ES-MS (electrospray ionization mass spectrometry), pooled and lyophilized for subsequent manipulation and/or ligation. For peptides containing protecting groups not removed in HF/p-cresol or piperidine/DMF, the protecting groups were retained, for instance, Acm-protected cysteines.

Boc-protected amino acids were obtained from Peptides International and Midwest Biotech. Fmoc-protected amino acids were obtained from Midwest Biotech or Nova Biochemicals. Trifluoroacetic acid (TFA) was obtained by Halocarbon. Other chemicals were from Fluka or Aldrich. Analytical and preparative HPLC were performed on a Rainin HPLC system with 214 nm UV detection using Vydac C4 analytical or preparative column. Peptide and protein mass spectrometry was performed on a Sciex API-I electrospray mass spectrometer.

Polymer Support Preparation

[0080] *AoA-Resin*: Aminooxy-functionalized cellulose-based affinity resin for oxime linkage was prepared as follows. Amino Spherilose affinity resin (ISCO, Lincoln, NE) was derivatized with Fmoc-aminooxy acetic acid and thoroughly washed with DMF. The Fmoc-aminooxyacetic acid was preactivated with DIC and N-hydroxysuccinamide for 45 minutes (for 9 mL of ISCO resin, 2 mmols each of NHS, DIC and Fmoc-AoA then allowed to couple to the resin for 45 minutes) and coupled to the amino Spherilose resin for 2-3 hours. After coupling was complete as monitored by ninhydrin assay, the resin was washed with DMF, the Fmoc group removed by treatment with 20% piperidine (2 x 3 minutes). After further washing with DMF, followed by DCM, the derivatized polymer support was thoroughly dried.

[0081] *Ni-NTA-IMAC Resin*: Obtained commercially from Pharmacia or Qiagen, and used as received from the manufacturer.

EXAMPLE 2: SOLID PHASE SYNTHESIS STRATEGY EMPLOYING DISPLACEABLE LINKER

[0082] A displaceable linker strategy was employed for solid-phase chemical ligation and its application to the synthesis of a test polypeptide, the mature full-length 129 amino acid residue sequence of human interleukin-4 (IL4). The mature full-length human IL4 contains six cysteines, and a single N-linked glycosylation site at asparagine position 38. The full-length IL4 polypeptide and the cysteine ligation sites employed for solid phase native chemical ligation is depicted below in **Table 1** as SEQ ID NO:1.

Table 1: Mature full-length Interleukin 4*

1 11 21 31 41

HKCDITLQEI IKTLNSLTEQ KTLCTELTVT DIFAASKNTT EKETFCRAAT

51 61 71 81 91

VLRQFYSHHE KDTRCLGATA QQFHRHKQLI RFLKRLDRNL

WGLAGLNSCP

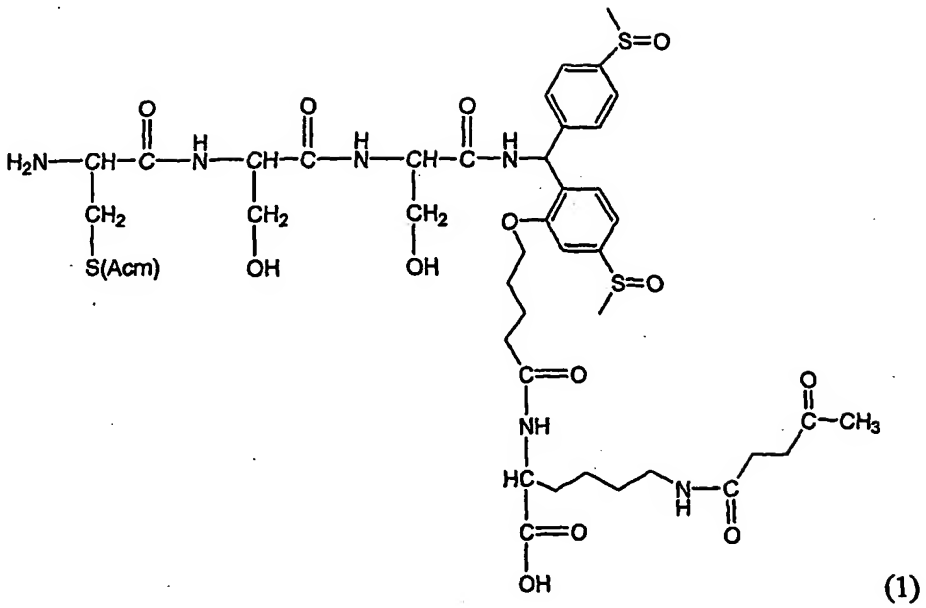
101 111 121

VKEANQSTLE NFLERLKTIM REKYSKCSS **(SEQ ID NO:1)**

*Single letter amino acid code; location of cysteine ligation sites denoted by boxed C cysteine residues.

[0083] In one approach, a displaceable oxime-forming linker was employed in combination with an amide-generating SCAL ("Safety-Catch Acid Cleavable") linker. The displaceable oxime-forming linker was designed to provide for attachment of the first peptide under mild conditions to a water-compatible spherical cellulose based support via a displaceable oxime linker, and the SCAL linker was designed to generate an amide capping group on the final full-length ligation product under acidic conditions following displacement of the oxime linker under mild conditions and its recovery from the support.

[0084] To form the initial IL4 peptide segment (containing IL4 amino acid residues 127-129) having an oxime bond with the support, a dual SCAL and a levulinic acid-modified lysine linker system was employed to form the compound having structure (1) depicted below.



(1)

[0085] As shown, structure (1) contains the extreme C-terminal IL4 peptide segment (127-129), and is alternatively denoted as having the amino acid sequence and linker attachment depicted below in SEQ ID NO:2.

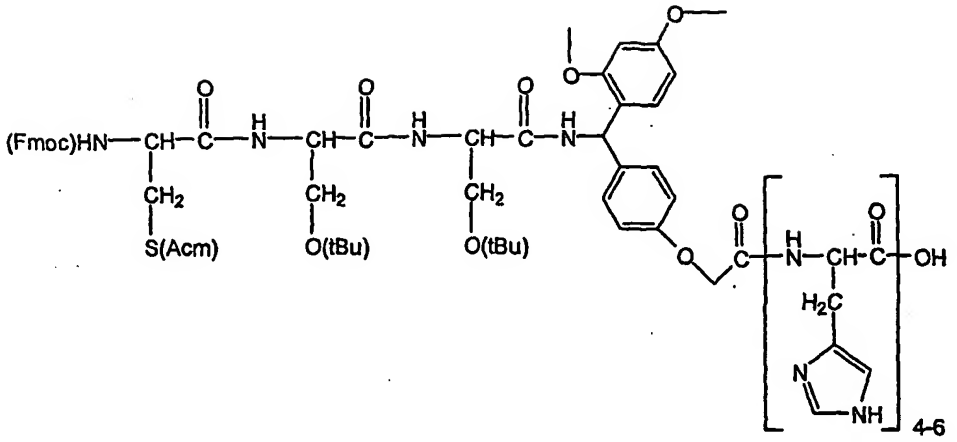
IL4:(^{Acm}127-129-$X^{SCAL}K^{lev}$):

C^{Acm}SS-$X^{SCAL}K^{lev}$ (SEQ ID NO:2)

where CSS corresponds to the C-terminal Cys-Ser-Ser residues 127-129 of human IL4 depicted in SEQ ID NO:1; C^{Acm} is a cysteine having its side chain thiol protected with an Acm-protecting group; X^{SCAL} is an amide-generating "safety-catch acid cleavable" linker; and K^{lev} is a modified lysine having its side chain epsilon nitrogen derivatized with levulinic acid.

[0086] In another approach, a displaceable metal chelator linker was employed in combination with an amide-generating, acid labile Rink amide linker. The displaceable metal chelator linker was designed to provide for attachment of the first peptide under mild conditions to a water-compatible spherical cellulose based support via a displaceable metal chelator linker, and the Rink linker was designed to generate an amide on the final full-length ligation product under acidic conditions following displacement of the metal chelator linker under mild conditions and its recovery from the support.

[0087] To form the initial IL4 peptide segment (containing IL4 amino acid residues 127-129) having a metal chelator linkage with the support, a dual Rink-Polyhistidine linker system was employed to form the compound having structure (2) depicted below.



(2)

[0088] As shown, structure (2) contains the extreme C-terminal IL4 peptide segment (127-129), and is alternatively denoted as having the amino acid sequence and linker attachment depicted below in SEQ ID NO:3 and SEQ ID NO:4.

IL4:(^{Acm}127-129-X^{Rink}HisTag):

C^{Acm}SS-X^{Rink}HHHH (SEQ ID NO:3)

C^{Acm}SS-X^{Rink}HHHHHH (SEQ ID NO:4)

where CSS corresponds to the C-terminal Cys-Ser-Ser residues 127-129 of human IL4 depicted in SEQ ID NO:1; C^{Acm} is a cysteine having its side chain thiol protected with an Acm-protecting group; X^{Rink} is an amide-generating, acid cleavable Rink amide linker; and "HisTag" corresponds to a polyhistidine amino acid sequence (His-His-His-His) or (His-His-His-His-His-His) for binding metal ions such as nickel.

[0089] For the remaining solid phase chemical ligation reactions, the following thioester peptides corresponding to IL4:(^{Acm}99-126^{COSR}) (SEQ ID NO:5), IL4:(^{Acm}46-98^{COSR}) (SEQ ID NO:6), IL4:(1-45^{COSR})^{a} (SEQ ID NO:7) and IL4:(1-45^{COSR})^{b} (SEQ ID NO:8) were prepared by highly optimized Boc SPPS and purified by HPLC before use in ligation reactions as described in **Example 1**. The amino acid sequences of the thioester peptides are depicted below.

IL4:(^{Acm}99-126^{COSR}):

C^{Acm}PVKE ANQST LENFL ERLKT IMREK YSK^{COSR} (SEQ ID NO:5)

IL4:(^{Acm}46-98^{COSR}):

C^{Acm}RAAT VLRQF YSHHE KDTRC LGATA QQFHR HKQLI RFLKR LDRNL WGLAG LNS^{COSR} (SEQ ID NO:6)

IL4:(1-45^{COSR})^{a}:

HKCDI TLQEI IKTLN SLTEQ KTLCT ELTVT DIFAA SKNTT EKETF^{COSR} (SEQ ID NO:7)

IL4:(1-45^{COSR})^{b}:

HKC^{Acm}DI TLQEI IKTLN SLTEQ KTLC^{Acm}T ELTVT DIFAA SKNTT EKETF^{COSR} (SEQ ID NO:8)

where C^{Acm} = Acm-protected cysteine; and COSR = thioester of a given C-terminal amino acid (e.g., a lysine-thioester in SEQ ID NO:5). The peptide IL4:(1-45^{COSR})^{a} was employed for oxime-forming linker approach, while the peptide IL4:(1-45^{COSR})^{b} (containing two internal Acm-protected cysteines) was employed for the metal chelator linker system to reduce side reactions with the free thiols.

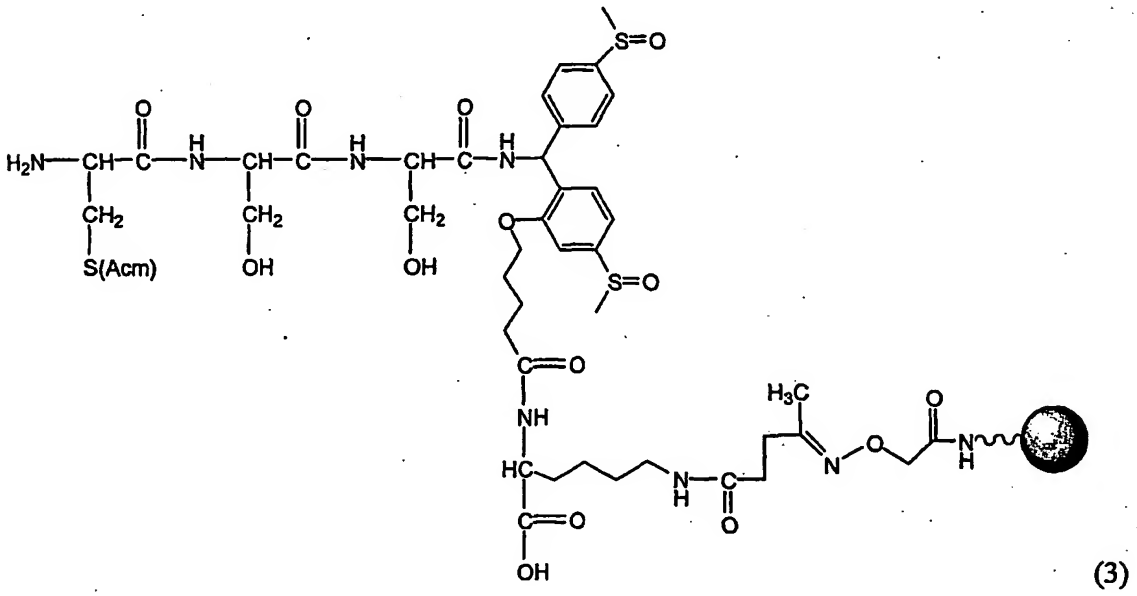
EXAMPLE 3: SYNTHESIS OF PEPTIDE IL4:(^{ACM}127-129)-X^{SCAL}-K^{OXIME} LINKER

[0090] Structure (1) of **Example 2** was accessed by Fmoc SPPS of the peptide (Fmoc)C^{Acm}S^{Bzl}S^{Bzl}-X^{SCAL}K^{Mtt} on a Leu-PAM resin (Applied Biosystems, 300 mg, 0.21 mmols), where the alpha-nitrogen of cysteine was protected with Fmoc, the thiol side chain cysteine was protected with Acm, the side chain hydroxyls of the serines were protected with benzyl (Bzl) groups, and the where epsilon nitrogen on the lysine side chain was protected with a Mtt protecting group. The peptide chain was constructed using manual Fmoc cycles described in example one.

[0091] The amino-terminal Fmoc group on the Cys residue was left intact, and the Mtt group was selectively removed from the Lys side chain by treatment with 2 x 1 minute TFA wash. Levulinic acid was coupled as the symmetric anhydride generated by treatment with DIC in DCM. After 30 minutes coupling the reaction was judged complete by ninhydrin test. The Fmoc group was then removed by treatment of the peptide with 20% piperidine in DMF for 2 x 10 minutes. The compound of structure (1) was then formed by cleavage and simultaneous deprotection (except for the Acm) from the PAM resin with a HF cocktail (5% p-cresol included as scavenger). The crude peptide was isolated and purified by HPLC as described in Example 1. As shown above, the C^{Acm}SS-X^{SCAL}K^{lev} construct depicted in structure (1) features a ketone functional group that provides a reactive handle for immobilization onto polymer supports modified with an aminooxy (AoA) group.

EXAMPLE 4: SYNTHESIS OF IL4:(^{ACM}127-129)-X^{SCAL}-K^{OXIME}-SUPPORT

[0092] The following sepharose resin-bound peptide was synthesized as intermediate structure (3) shown below:



(3)

[0093] Structure (3) was made by reacting structure (1) dissolved in pH 3.5 sodium acetate buffer and an excess of AoA-Resin as follows. Approximately 40 mg of the ketone containing peptide of structure (1) was dissolved in 12 mL of a solution containing 20% acetonitrile and 0.1 M sodium acetate at pH 3.5, and then added to 9 ml of AoA-resin slurry from stock and allowed to react at room temperature for 16 hours. Synthesis of immobilized structure (3) was monitored by periodically removing small aliquots (~10 µl) of the supernatant and observing the disappearance of structure (1) from solution by HPLC. Oxime bond formation was typically found to be complete overnight. It was confirmed that structure (3) was present by removing an analytical sample of resin. To prepare the resin of the reaction product in **Example 4** for the first native chemical ligation reaction, the Acm-protecting group of structure (3) was removed by washing the peptide-bound resin with 3% aqueous acetic acid followed by treatment for 2 x 60 minutes with a solution of mercury(II) acetate (5 mg/ml) in the acetate buffer at pH 3.5. After thorough washing of the resin with the acetic acid solution, the peptide-resin is then treated with 20% β-mercaptoethanol in 6 M guanidinium chloride, then washed with 6 M guanidinium chloride/sodium phosphate (pH 7).

[0094] After removal of the Acm-protecting group by treatment with mercuric acetate, the resin-bound peptide IL4:(127-129)-$X^{SCAL}K^{OXIME}$-Support was ready for elongation by native

chemical ligation with the first incoming thioester peptide. In particular, segment IL4:(^{Acm}99-126^{COSR}) was ligated to resin-bound segment IL4:(127-129)-$X^{SCAL}K^{OXIME}$-Support to give the first ligation product IL4:(Acm99-129)-$X^{SCAL}K^{OXIME}$-Support as follows. 22 mg of the incoming peptide IL4:(Acm99-126^{COSR} was dissolved in 300 mM sodium phosphate buffer (pH 7.5) containing 6 M guanidinium chloride (2-4 mM), and 0.5% thiophenol (~ 5 mM peptide concentration). Before the addition of the incoming second peptide, the polymer-support resin (4.5 mL of resin) was thoroughly washed in the same buffer used to dissolve the second peptide. The mixture was allowed to equilibrate and react at room temperature for 48 hours. After ligation, the polymer-support resin was thoroughly washed with 300 mM sodium phosphate buffer (pH 7.5) containing 6 M guanidinium chloride (2-4 mM), then with 3% acetic acid]. The identity of the first on-resin ligation product containing IL4 residues 99-129 was confirmed by displacement of the resulting bound IL4 fragment by aminooxy acetic acid treatment (1 M aminooxy acetic acid in water, 1 hr at room temperature). The ligation reaction was found to proceed smoothly overnight. In a typical experiment, displaced intermediate IL4:(Acm99-129)-$X^{SCAL}K^{OXIME}$ elutes as a single peak on analytical HPLC with an observed mass by ES-MS of 4522 amu.

EXAMPLE 6: SOLID PHASE CHEMICAL LIGATION OF IL4:(^{ACM}46-98^{COSR}) TO IL4:(99-129)-X^{SCAL}-K^{OXIME}-SUPPORT

[0095] To prepare the resin of the reaction product in **Example 5** for the second native chemical ligation reaction, the Acm-protecting group on the N-terminal cysteine of IL4:(^{Acm}99-129)-X^{SCAL}-K^{OXIME}-Support was removed as described in **Example 5**. Following Acm removal, native chemical ligation of the thioester peptide IL4:(^{Acm}46-98^{COSR}) with resin-bound IL4:(99-129)-$X^{SCAL}K^{OXIME}$-Support generated IL4:(^{Acm}46-129)-$X^{SCAL}K^{OXIME}$-Support. The ligation reaction was performed by treating deprotected peptide resin (4.5 ml) with a solution of the incoming peptide (15 mg) in 0.5 ml of 6 M guanidinium chloride containing 300 mM sodium phosphate pH 7.5, 0.5 % thiophenol as described in **Example 5**. When displaced from the resin with 1 M AoA as described above, this product also elutes as single peak in analytical HPLC, with an observed mass by ES-MS of 10,867 amu.

EXAMPLE 7: SOLID PHASE CHEMICAL LIGATION OF IL4:(1-45^{COSR})^{a} TO IL4:(46-129)-X^{SCAL}-K^{OXIME}-SUPPORT

[0096] To prepare the resin of the reaction product in **Example 6** for the final native chemical ligation reaction, the Acm-protecting group on the N-terminal cysteine of IL4:(^{Acm}46-129)-X^{SCAL}-K^{OXIME}-Support was removed as described in **Example 5**. Following

Acm removal, on-resin assembly of the IL4 polypeptide chain was completed by reaction of the thioester peptide IL4:(1-45^{COSR})^{a} with the Acm-deprotected N-terminal cysteine of the IL4:(46-129)-X^{SCAL}K^{OXIME}-Support material. The ligation reaction was performed by treating deprotected peptide resin with a solution of 10 mg of the incoming peptide in 200 µl of 6 M guanidinium chloride containing 300 mM sodium phosphate pH 7.5, 0.5 % thiophenol as described in **Example 5**. After reacting overnight, an analytical sample of peptide was displaced from the resin as described above using 1 M AoA as described above and analyzed by HPLC and ES-MS. HPLC analysis showed recovery of the desired target product: IL4:(1-129)-X^{SCAL}K^{OXIME}, MW = 15,810 Da (**FIG.2A**).

[0097] The remaining IL4:(1-129)-X^{SCAL}K^{OXIME}-Support material was then reacted with 1 M AoA as described above to displace the ligated IL4 polypeptide from the resin. The resulting SCAL-Oxime linker-modified protein IL4:(1-129)-X^{SCAL}K^{OXIME} was desalted on a semi-preparative reverse phase HPLC column. Cleavage of the SCAL linker resulted in removal of the SCAL-Oxime linker system, leaving behind the amide at Ser129. To effect this cleavage, desalted IL4:(1-129)-X^{SCAL}K^{OXIME} was treated with TFA (1.5 mL, containing 0.2 mL thioanisole, 0.1 mL ethanedithiol) with 0.2 mL of bromotrimethylsilane for 1/2 hour at 0° C. The peptide was then precipitated with cold ether, and the residue dissolved in a minimal amount of 50% water/acetonitrile containing 0.1 % TFA. After lyophilization, HPLC and ES-MS analysis showed that full-length IL4:(1-129) (**SEQ ID NO:1**) was generated by cleavage of the SCAL linker, MW = 14,960 Da (**FIG.2B**). The desired full-length polypeptide was then isolated and purified using semi-preparative HPLC.

EXAMPLE 8: SYNTHESIS OF IL4:(FMOC)CYS^{ACM}S^{TBU}S^{TBU}-X^{RINK}HISTAG LINKER

[0098] Structure (2) of **Example 2** was accessed by Fmoc SPPS of the peptide IL4:(Fmoc)C^{Acm}S^{tBu}S^{tBu}-X^{Rink}HisTag on a Sasrin resin as follows. 98 mg of Sasrin resin (0.1 mmol) was washed with DMF for one minute. 12.22 mg of DMAP (0.1mmol) and 13.5 mg of HOBT were dissolved in 3 ml DMF. The resulting solution was used to dissolve 1 mmol of Fmoc-His. Then 0.15 ml of DIC was added to the solution described above. This Fmoc-His solution was added to the dry Sasrin resin and the reaction was allowed proceed at room temperature for one hour. To remove the Fmoc protecting group, the resin bound peptide was treated with 20% piperidine in DMF 2 x 3 minutes. The resin was then washed with DMF.

[0099] For coupling the remaining histidines, for each coupling reaction, 1 mmol of Fmoc-His was dissolved in 1.8 ml of 0.5 M HBTU in DMF and 0.5 ml of DIEA, and the resulting solution was added to the resin. Each coupling reaction was run for 40 minutes at room temperature. After each coupling, the resin washed with DMF, followed by removal of the Fmoc protecting group with 20% piperidine in DMF (2 X 3 minutes for each time), and finally washing the deprotected resin again with DMF. The process for coupling the remaining histidine residues described above was repeated two or four more times to generate either a 4-His tag, or a 6-His tag.

[0100] Following the histidine additions, the Rink-amide linker was added to complete assembly of the linker system as follows. 1 mmol of Fmoc-Rink amide linker was dissolved in 1.8 ml of HBTU and 0.5 ml of DIEA, and the resulting solution was added to the resin. The reaction was run for 40 minutes at room temperature. Then the resin was washed with DMF. The Fmoc protecting group was removed with 20% piperidine in DMF twice, three minutes for each time. Then the resin was washed with DMF.

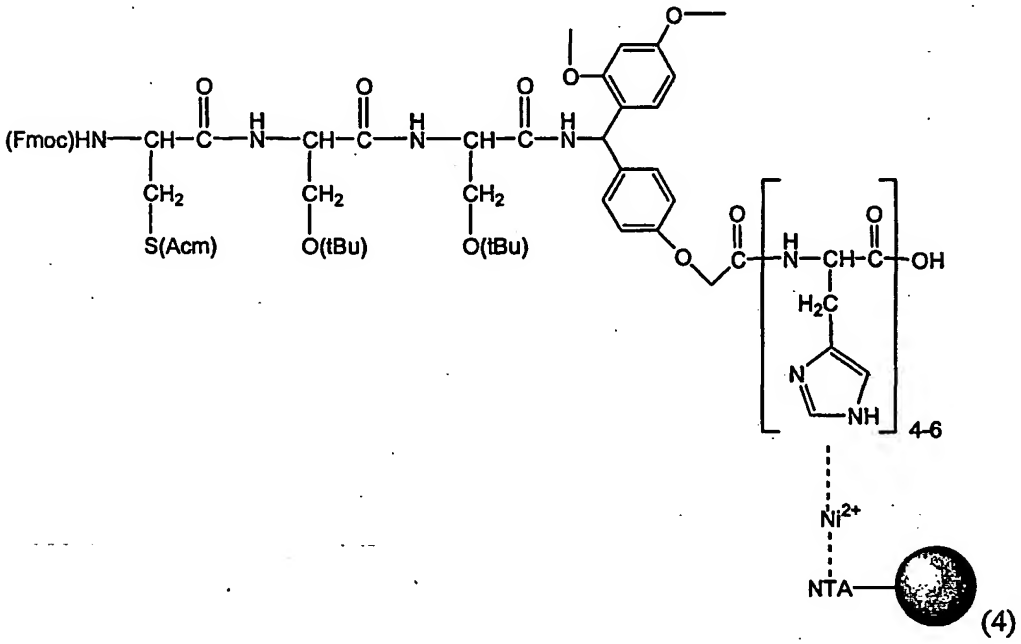
[0101] The three pendant C-terminal amino acids of the full-length IL4 sequence were then coupled to the unprotected Rink-amide linker as follows. 1 mmol of Fmoc-Ser(tBu) was dissolved in 1.8 ml of HBTU and 0.5 ml of DIEA, and the resulting solution was added to the resin. Each reaction was run for 40 minutes at room temperature. Then the resin was washed with DMF. The Fmoc protecting group was removed with 20% piperidine in DMF twice, three minutes for each time. Then the resin was washed with DMF. The process described above was repeated to add the second serine residue, followed by addition of Fmoc-Cys(Acm).

[0102] After the above coupling stages, to remove the peptide IL4:(Fmoc)$C^{Acm}S^{tBu}S^{tBu}$-X^{Rink}HisTag from the Sasrin resin, 2% TFA/DCM was added to the resin bound peptide and the reaction was allowed to run at room temperature for 45 minutes. Thioester peptides employed for subsequent ligations were prepared as described above in **Example 2**.

EXAMPLE 9: BINDING OF IL4:(FMOC)CYS^{ACM}S^{TBU}S^{TBU}-X^{RINK}HISTAG TO NI-NTA RESIN

[0103] Structure (4) shown below was generated as follows. 3 mL of Ni-NTA resin from **Example 1** was washed with distilled water (50 mL). 3 mg of the peptide IL4:(Fmoc)Cys^{Acm}S^{tBu}S^{tBu}-X^{Rink}HisTag from **Example 8** was dissolved in 300 µL of 6 M guanidinium chloride, pH 7.5. The peptide solution was added to the Ni-NTA resin, then the

resulting resin was washed with 6 M guanidinium chloride, pH 7.5 (50 mL), and the eluant was analyzed by HPLC. No IL4:(Fmoc)Cys^{Acm}S^{tBu}S^{tBu}-X^{Rink}HisTag peptide was detected, indicating that all of the peptide bound to the Ni-NTA-resin.



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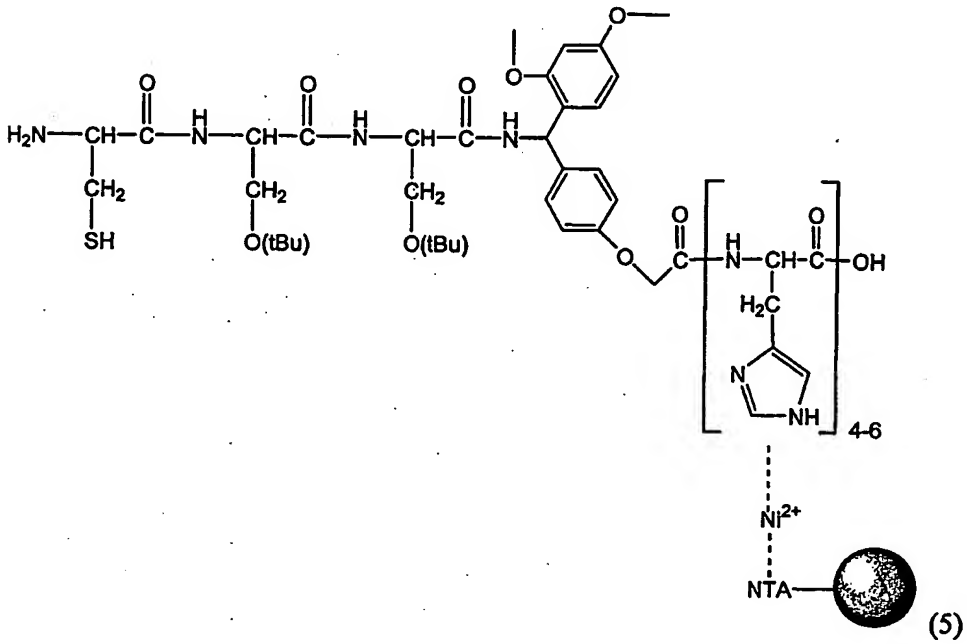
EXAMPLE 10: ESTABLISHMENT OF REVERSIBLE BINDING OF IL4:(FMOC)CYS^{ACM}S^{TBU}S^{TBU}-X^{RINK}HISTAG LINKER

[0104] The bound peptide of **Example 9** was eluted from the resin using 0.5 M imidazole solution in water. The eluant was analyzed by HPLC and a single peak with retention time 16.08 minutes was found, corresponding to the IL4:(Fmoc)Cys^{Acm}S^{tBu}S^{tBu}-X^{Rink}HisTag peptide (ES-MS, MW = 1549 amu).

EXAMPLE 11: REMOVAL OF FMOC AND ACM PROTECTING GROUPS FROM IL4:(FMOC)CYS^{ACM}S^{TBU}S^{TBU}-X^{RINK}HISTAG-NI-NTA-SUPPORT

[0105] The resin-bound peptide IL4:(127-129)-X^{Rink}HisTag-Ni-NTA-Support, having structure (5) shown below, was generated as follows. The Acm-thiol protecting group of the product of **Example 9** (structure (4)) was removed by adding 3 mL of 6.6 mg/mL $Hg(OAc)_2$ in 0.1 M acetate buffer, pH 6 to the peptide bound resin. The reaction was allowed to proceed for an hour. The resin was washed with 0.1 M acetate buffer, pH 6. To remove the Fmoc-amino protecting group from the N-terminal cysteine of this product, the resin bound peptide was treated with 20% piperidine in DMF twice, three minutes for each time. Then, the resin was treated with the solution of 10% β-mercaptoethanol in 6 M guanidinium

chloride, pH 7.2 (2 mL) and very small amount of TCEP (0.3 mg) for 0.5 hour. The deprotected peptide was then removed from the resin using the elution conditions described above in the step of reversible binding of His-Tagged peptides. Analysis of the eluant by HPLC found two peaks with retention times of 19.7 and 20.1 min. ES-MS analysis of these peaks showed that these were the deprotected peptide products (MW = 1255 amu). The different elution times were attributed to diastereomerism at the chiral center of the Rink linker. The serine side chain tBu protecting groups were retained for convenience, as they were removed under the conditions for removal of the Rink linker from the final ligation product as described below.



(5)

EXAMPLE 12: SOLID PHASE CHEMICAL LIGATION OF IL4:(^{ACM}99-126^{COSR}) TO IL4:(127-129)-X^{RINK}HISTAG-NI-NTA-SUPPORT

[0106] For the first chemical ligation reaction, peptide segment IL4:(^{Acm}99-126^{COSR}) was ligated to structure (5) of **Example 11** to give the first ligation product IL4:(^{Acm}99-129)-X^{Rink}HisTag-Ni-NTA-Support as follows. 3 mg of peptide IL4:(^{Acm}99-126^{COSR}) was dissolved in 200 µL of 6M guanidinium chloride, pH 7.5. The resulting solution was added to the resin and then 1 µL of thiophenol was added to the reaction solution. The reaction was run overnight and the resin was washed with of 6M guanidinium chloride, pH 7.2 (40 mL). HPLC analysis showed a peak of the product with the retention time 25.02 mins and ES-MS found that the product molecular weight was 4664 amu.

EXAMPLE 13: REMOVAL OF ACM PROTECTING GROUP FROM IL4:(^{Acm}99-129)-X^{Rink}HisTag-Ni-NTA-Support

[0107] To remove the N-terminal Acm group from the ligation product of **Example 12**, 3 mL of 6.6 mg/mL $Hg(OAc)_2$ in 0.1 M acetate buffer, pH 6 was added to the peptide bound resin. The reaction was allowed to proceed for an hour. The resin was washed with 20 mL 0.1 M acetate buffer, pH 6. The resin was treated with 2 mL of 10% β-mercaptoethanol in 6 M guanidinium chloride, pH 7.2 and very small amount of TCEP (0.3 mg) for 0.5 hour and then washed with 6 M guanidinium chloride, pH 7.2.

EXAMPLE 14: SOLID PHASE CHEMICAL LIGATION OF IL4:(^{Acm}46-98^{COSR}) TO IL4:(99-129)-X^{Rink}HisTag-Ni-NTA-Support

[0108] The next peptide ligation reaction was carried out on the product of **Example 13** as follows. 2.5 mg of the thioester peptide IL4:(^{Acm}46-98^{COSR}) was dissolved in 200 µL of 6M guanidinium chloride, pH 7.5. The resulting solution was added to the resin and then 1µL of thiophenol was added to the reaction solution. The reaction was run overnight and the resin was washed with 40 mL of 6 M guanidinium chloride, pH 7.2. HPLC analysis found a peak of the product with the retention time 26.4 min. ES-MS analysis of the product showed that the molecular weight of the product is 11,010 amu.

EXAMPLE 15: REMOVAL OF ACM PROTECTING GROUPS FROM IL4:(^{Acm}46-129)-X^{Rink}HisTag-Ni-NTA-Support

[0109] To remove the two Acm groups from the ligation product of **Example 14**, 3 mL of 6.6 mg/mL $Hg(OAc)_2$ in 0.1M acetate buffer, pH 6 were added to the peptide-bound resin. The reaction was allowed to proceed for an hour. The resin was washed with 0.1 M acetate buffer, pH 6. The resin was washed with 20 mL 0.1 M acetate buffer, pH 6. Then, the resin was treated with 2 mL of 10% β-mercaptoethanol in 6 M guanidinium chloride, pH 7.2 and very small amount of TCEP (0.3 mg) for 0.5 hour and then washed with 6 M guanidinium chloride, pH 7.2. Analytical HPLC found a peak of the product with a retention time of 26.3 min. ES-MS analysis found a molecular weight of 10,868 amu for the product.

EXAMPLE 16: SOLID PHASE CHEMICAL LIGATION OF IL4:(1-45^{COSR})^{b} TO IL4:(46-129)-X^{Rink}HisTag-Ni-NTA-Support

[0110] The final on-resin peptide ligation was performed on the product of **Example 15** as follows. 1.5 mg of the peptide thioester segment IL4:(1-45^{COSR})^{b} was dissolved in 100 µL of 6M guanidinium chloride, pH 7.2. The resulting solution was added to the resin and then 0.5

μL of thiophenol was added to the reaction solution. The reaction was run for 3 hours. A small analytical sample of the support bound, full-length ligation product was released from the support by displacement with imidazole-containing buffer as described above. The eluant containing the released ligation product was readily separated from the resin, and HPLC analysis of the eluant showed a peak corresponding to the full-length ligation product with a retention time of 28.1 minutes and ES-MS analysis of the product found a molecular weight of 16,092 amu.

EXAMPLE 17: REMOVAL OF INTERNAL ACM PROTECTING GROUPS FROM IL4:(1-129)-X^{Rink}HisTag-Ni-NTA-SUPPORT

[0111] To remove two remaining internal Acm protecting groups from the last peptide ligation, 3 mL of 6.6 mg/mL $Hg(OAc)_2$ in 0.1 M acetate buffer, pH 6 was added to the peptide resin produced in **Example 16**. The reaction was allowed to proceed for one hour. The resin was washed with 20 mL 0.1 M acetate buffer, pH 6. A small analytical sample of the support resin was treated with 2 mL of 10% β-mercaptoethanol in 6 M guanidinium chloride, pH 7.2 and very small amount of TCEP for a half hour and then washed with 6 M guanidinium chloride, pH 7.2. Analytical HPLC showed a peak with the retention time 28.2 minutes corresponding to the product. ES-MS analysis found a molecular weight of 15,952 amu for the product, full-length IL-4 with the Rink and HisTag linkers intact. These data confirmed that the two internal Acm groups on the last peptide segment employed for ligation were removed.

EXAMPLE 18: DISPLACEMENT OF FULL-LENGTH IL4:(1-129)-X^{Rink}HisTag LIGATION PRODUCT FROM THE Ni-NTA-SUPPORT

[0112] The full-length, product from **Example 17** was released from the support by displacement with imidazole containing buffer as described above in **Example 10**. Specifically, the peptide resin product of **Example 17** was treated with 2 mL of 10% β-mercaptoethanol in 6 M guanidinium chloride, pH 7.2 and very small amount of TCEP (0.3 mg) for a half hour and then washed with 6 M guanidinium chloride, pH 7.2, followed by release of the product using 0.5M imidazole solution in water. Analytical HPLC showed a peak with the retention time 28.2 minutes corresponding to the product (See, **FIG.4A**). ES-MS analysis found a molecular weight of 15,952 amu for the released full-length IL-4 ligation product with the Rink and HisTag linkers intact.

EXAMPLE 19: CLEAVAGE OF THE RINK-HISTAG LINKER, AND FORMATION OF FULL-LENGTH IL4 (1-129)

[0113] 0.3 mg of full-length ligation product from **Example 18** bearing the Rink-HisTag linker system were dissolved in 1 mL of 90% TFA with 5% water and 5% EDT (1,2-ethanedithiol). The reaction was run for 45 min. The solution was dried and the product re-dissolved in 1 mL of 20% β-mercaptoethanol in 6 M guanidinium chloride, at pH 7.2 and very small amount of TCEP (0.3 mg) for 2 hours. Analytical HPLC showed a peak with the retention time 29.8 minutes corresponding to the product (See, **FIG.4B**). ES-MS analysis found a molecular weight of 14,992 amu, confirming that this was the desired product, full-length IL4 polypeptide with the Rink and HisTag linkers removed.

EXAMPLE 20: FOLDING OF FULL-LENGTH IL4 (1-129)

[0114] Full-length ligation product IL4 (1-129) from **Example 19** was folded by dissolving 1 mg of the unfolded material in 1 ml of 6 M guanidinium chloride buffer with 100 mM Tris, 1 mM cysteine, and 0.2 mM cystine, at pH 8. The resulting solution was injected into a dialysis cassette (3500 MWCO, 0.1-0.5 ml). Then the cassette was put into 1000 ml of 0.5 M guanidinium chloride buffer with 100 mM Tris, 1 mM cysteine, and 0.2 mM cystine, at pH 8. The result was checked after overnight folding. Analytical HPLC showed a peak with the retention time 27.892 minutes for the folding product. ES-MS analysis found a molecular weight of 14,988 amu, indicative of the expected mass shift for disulfide formation. Circular dichroism (CD) also was performed following standard protocols and confirmed the formation of secondary structure, and thus folding of the protein.

[0115] The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

WHAT IS CLAIMED IS:

1. A method for aqueous-compatible solid phase chemical ligation, said method comprising:

(a) ligating under aqueous conditions a first polymer to a second polymer to form a ligation product bound to an aqueous-compatible solid support, the first and second polymers having mutually reactive chemical groups capable of chemoselective chemical ligation under aqueous conditions, the first polymer being attached to the solid support through a linker system comprising a displaceable linker; and

(b) displacing the displaceable linker so as to release the ligation product from the solid support.

2. The method of claim 1, which further comprises:

(c) separating the ligation product produced in step (b) from the solid support.

3. The method of claim 1, wherein said chemoselective chemical ligation is selected from the group consisting of amide-forming chemical ligation, oxime-forming chemical ligation, hydrazone-forming chemical ligation, thiazolidine-forming chemical ligation, thioester-forming chemical ligation, selenolester-forming chemical ligation, thioether-forming chemical ligation, and oxazolidine-forming chemical ligation.

4. The method of claim 1, wherein said first and second polymers are selected from the group consisting of peptides, polypeptides, nucleic acids, lipids, carbohydrates and synthetic polymers.

5. The method of claim 1, wherein said displaceable linker is selected *from the group consisting of a chemically displaceable linker and an enzymatically* displaceable linker.

6. The method of claim 5, wherein said chemically displaceable linker is selected from the group consisting of oxime linker, hydrazone linker, diol linker, photolabile linker, reducible linker, and metal chelator linker.

7. The method of claim 5, wherein said enzymatically displaceable linker is cleavable by a hydrolytic enzyme.

8. The method of claim 6, wherein said displaceable linker is an oxime linker and said displacing comprises contacting said displaceable linker with a compound comprising an aminooxy group.

9. The method of claim 6, wherein said displaceable linker is a hydrazone linker and said displacing comprises contacting said hydrazone linker with a compound comprising a hydrazine.

10. The method of claim 6, wherein said displaceable linker is a diol linker and said displacing comprises contacting said diol linker with a compound comprising a diol.

11. The method of claim 6, wherein said displaceable linker is a photolabile linker and said displacing comprises exposing said photolabile linker to a wavelength of light that cleaves said photolabile linker.

12. The method of claim 6, wherein said displaceable linker is a reducible linker and said displacing comprises contacting said reducible linker with a compound comprising a reducing agent.

13. The method of claim 6, wherein said displaceable linker is a metal chelator linker and said displacing comprises one or more of (i) contacting said metal chelator linker with a compound comprising a metal chelator, and (ii) adjusting a condition that displaces the displaceable linker, wherein said condition is selected from the group consisting of pH and salt concentration.

14. The method of claim 1, wherein said linker system comprises a second linker that joins said displaceable linker to said first polymer, and wherein said second linker is cleavable under conditions orthogonal to said displaceable linker.

15. The method of claim 14, wherein said method further comprises:

(d) cleaving the second linker from the ligation product produced in step (c); and

(e) recovering the ligation product produced in step (d).

16. The method of claim 14, wherein said second linker is cleavable with a reagent selected from the group consisting of acid, hydrogen-generating catalyst, and base.

17. The method of claim 16, wherein said second linker is selected from the group consisting of an acid cleavable linker, a hydrogen-generating catalyst cleavable linker, and a base cleavable linker.

18. The method of claim 16, wherein said acid is selected from the group consisting of trifluoroacetic acid and anhydrous hydrogen fluoride.

19. The method of claim 16, wherein said base is sodium hydroxide.

20. The method of claim 16, wherein said hydrogen-generating catalyst is a reducing metal.

21. The method of claim 1, wherein said aqueous conditions comprise water and an excipient selected from the group consisting of buffer, ligation catalyst, denaturant, lipid, detergent and chaotrope.

22. The method of claim 1, wherein said aqueous conditions comprises a mixture of water and an organic solvent.

23. The method of claim 1, wherein said aqueous-compatible solid support is a support that swells in water or in mixed water-organic conditions.

24. The method of claim 23, wherein said support that swells in water comprises a polymer selected from the group consisting of cellulose, agarose, dextran and polyethylene glycol.

25. The method of claim 1, wherein said aqueous-compatible solid support is a support that does not swell in water or in mixed water-organic conditions.

26. The method of claim 25, wherein said support that does not swell in water or in mixed water-organic conditions is a controlled pore glass support.

27. The method of claim 1, wherein said first polymer comprises the reaction product of one or more chemoselective ligations.

28. The method of claim 1, wherein said second polymer comprises the reaction product of one or more chemoselective ligations.

29. A composition comprising a partially or fully unprotected polymer having a first end attached to an aqueous-compatible support through a linker system comprising a displaceable linker selected from the group consisting of a hydrazone linker, a diol linker, a photolabile linker, a reducible linker, a metal chelator linker and a biologically cleavable linker, and a second end bearing a protected or unprotected chemoselective reactive group capable of chemoselective chemical ligation with a mutually reactive chemoselective reactive group.

30. The composition of claim 29, wherein said chemoselective chemical ligation is selected from the group consisting of amide-forming chemical ligation, oxime-forming chemical ligation, hydrazone-forming chemical ligation, thiazolidine-forming chemical ligation, thioester-forming chemical ligation, selenolester-forming chemical ligation, thioether-forming chemical ligation, and oxazolidine-forming chemical ligation.

31. The composition of claim 29, wherein said polymer is selected from the group consisting of peptides, polypeptides, nucleic acids, lipids, carbohydrates and synthetic polymers.

32. The composition of claim 29, wherein said displaceable linker is a hydrazone linker that is displaceable with a compound comprising a hydrazine.

33. The composition of claim 29, wherein said displaceable linker is a diol linker that is displaceable with a compound comprising a diol.

34. The composition of claim 29, wherein said displaceable linker is a photolabile linker that is displaceable with light.

35. The composition of claim 29, wherein said displaceable linker is a reducible linker that is displaceable under reducing conditions.

36. The composition of claim 29, wherein said displaceable linker is a metal chelator linker that is displaceable with one or more of (i) a compound comprising a metal chelator, and (ii) a condition selected from the group consisting of pH and salt concentration.

37. The composition of claim 29, wherein said linker system comprises a second linker that joins said displaceable linker to said first polymer, and wherein said second linker is cleavable under conditions orthogonal to said displaceable linker.

38. The composition of claim 37, wherein said second linker is cleavable with a reagent selected from the group consisting of acid, hydrogen-generating catalyst, and base.

39. The composition of claim 38, wherein said acid is selected from the group consisting of trifluoroacetic acid and anhydrous hydrogen fluoride.

40. The composition of claim 38, wherein said base is sodium hydroxide.

41. The composition of claim 38, wherein said hydrogen-generating catalyst is a reducing metal.

42. The composition of claim 29, wherein said composition comprises water and an excipient selected from the group consisting of buffer, ligation catalyst, denaturant, lipid, detergent and chaotrope.

43. The composition of claim 29, wherein said aqueous conditions comprises a mixture of water and an organic solvent.

44. The composition of claim 29, wherein said aqueous-compatible solid support is a support that swells in water or in mixed water-organic conditions.

45. The composition of claim 44, wherein said support that swells in water comprises a polymer selected from the group consisting of cellulose, agarose, dextran and polyethylene glycol.

46. The composition of claim 29, wherein said aqueous-compatible solid support is a support that does not swell in water or in mixed water-organic conditions.

47. The composition of claim 46, wherein said support that does not swell in water or in mixed water-organic conditions is a controlled pore glass support.

48. The composition of claim 29, wherein said polymer comprises the reaction product of one or more chemoselective ligations.

49. An isolated composition comprising a partially or fully unprotected polymer that is substantially pure and free of a solid support, said composition comprising the reaction product of a chemical ligation reaction and having a first end attached to a displaceable linker through a second linker that is cleavable under conditions orthogonal to said displaceable linker.

50. The composition of claim 49, wherein said composition comprises water and an excipient selected from the group consisting of buffer, ligation catalyst, denaturant, lipid, detergent and chaotrope.

51. The composition of claim 49, wherein said composition is a lyophilized powder.

52. The composition of claim 49, wherein said partially or fully unprotected polymer that is substantially monodisperse.

53. A kit comprising a container having deposited therein a composition according to any one of claims 29 to 52.

54. A kit comprising a protocol for carrying out a method according to any one of claims 1 to 28.

55. A kit comprising a container having deposited therein a composition according to any one of claims 29 to 52, and a protocol for carrying out a method according to any one of claims 1 to 28.

FIGURE 1

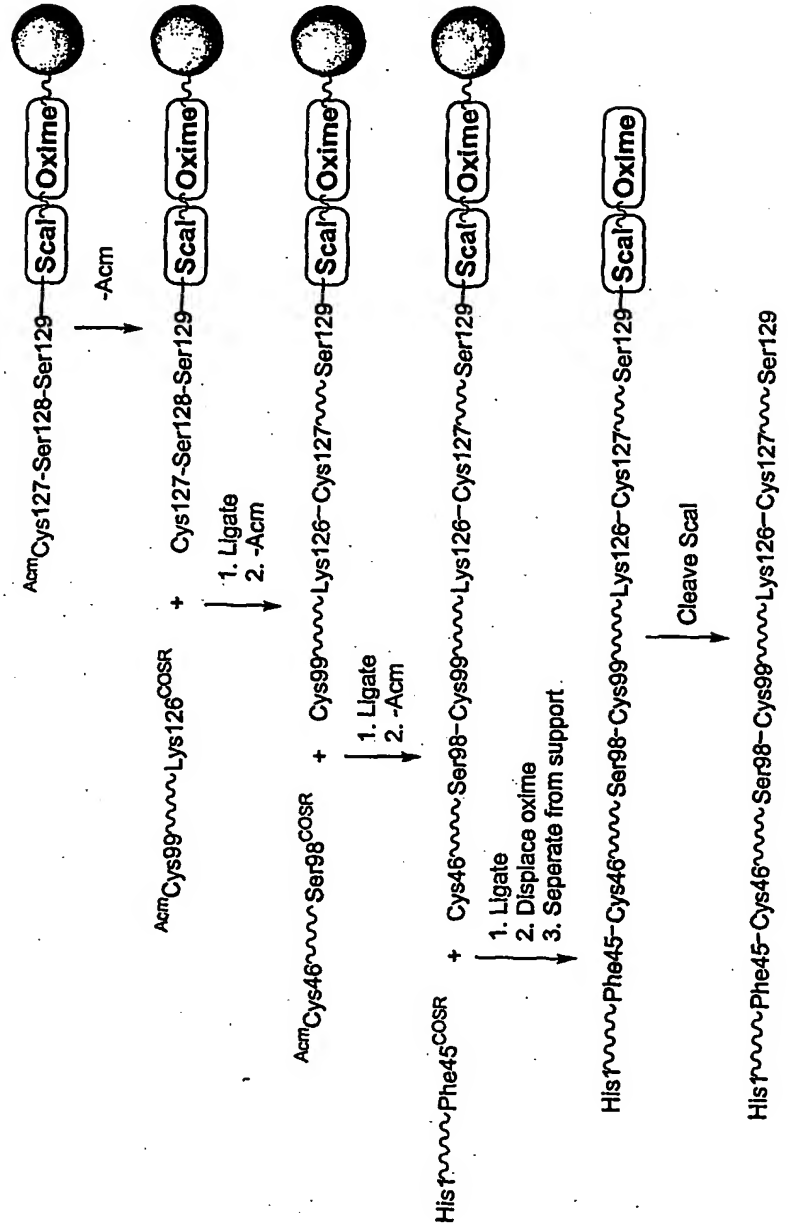

AcmCys127-Ser128-Ser129
ScaI
Oxime
-Acm
AcmCys99~~~Lys126COSR
+
Cys127-Ser128-Ser129
1. Ligate
2. -Acm
AcmCys46~~~Ser98COSR
+
Cys99~~~Lys126-Cys127~~~Ser129
1. Ligate
2. -Acm
His1~~~Phe45COSR
+
Cys46~~~Ser98-Cys99~~~Lys126-Cys127~~~Ser129
1. Ligate
2. Displace oxime
3. Seperate from support
His1~~~Phe45-Cys46~~~Ser98-Cys99~~~Lys126-Cys127~~~Ser129
Cleave ScaI
His1~~~Phe45-Cys46~~~Ser98-Cys99~~~Lys126-Cys127~~~Ser129

FIGURE 2

FIG.2A

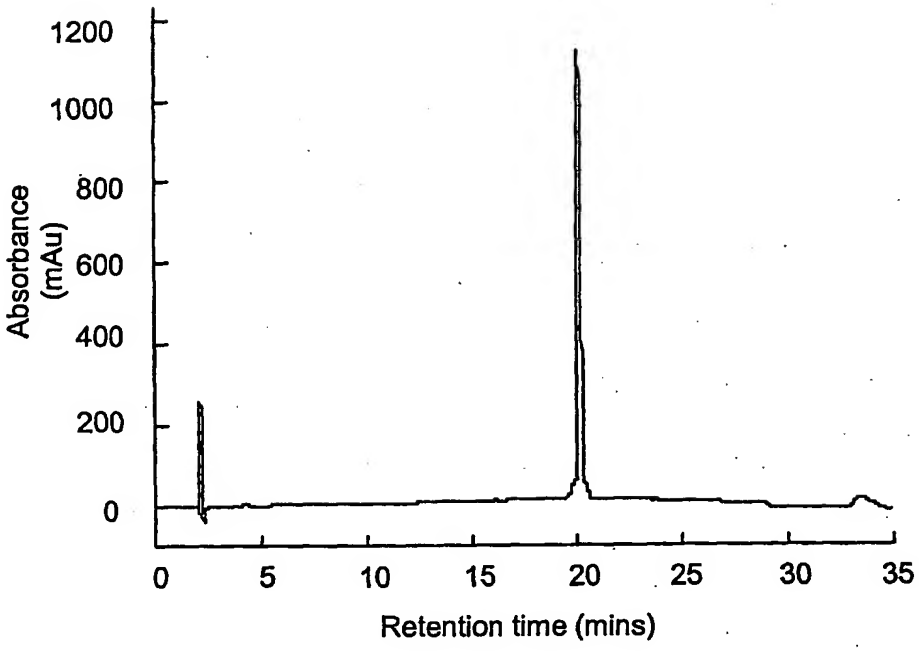


FIG.2B

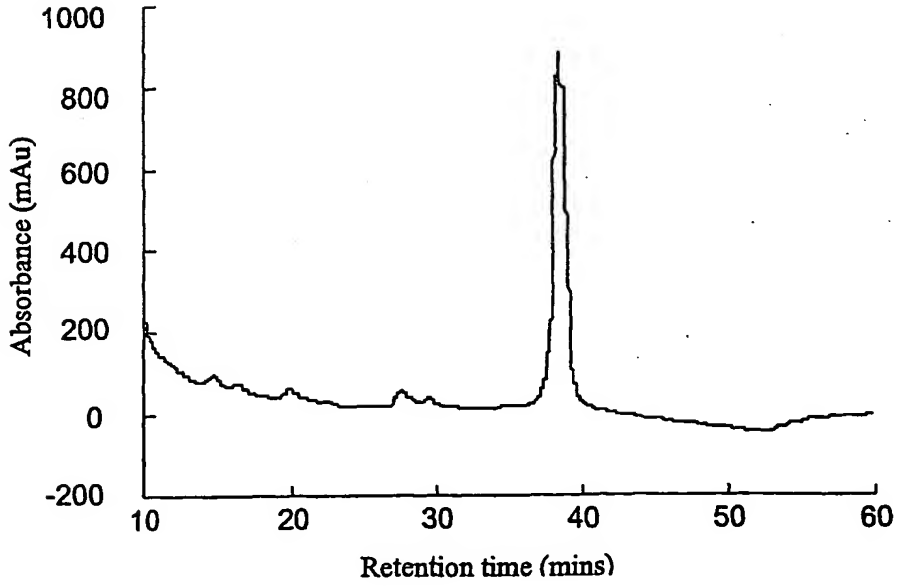


FIGURE 3

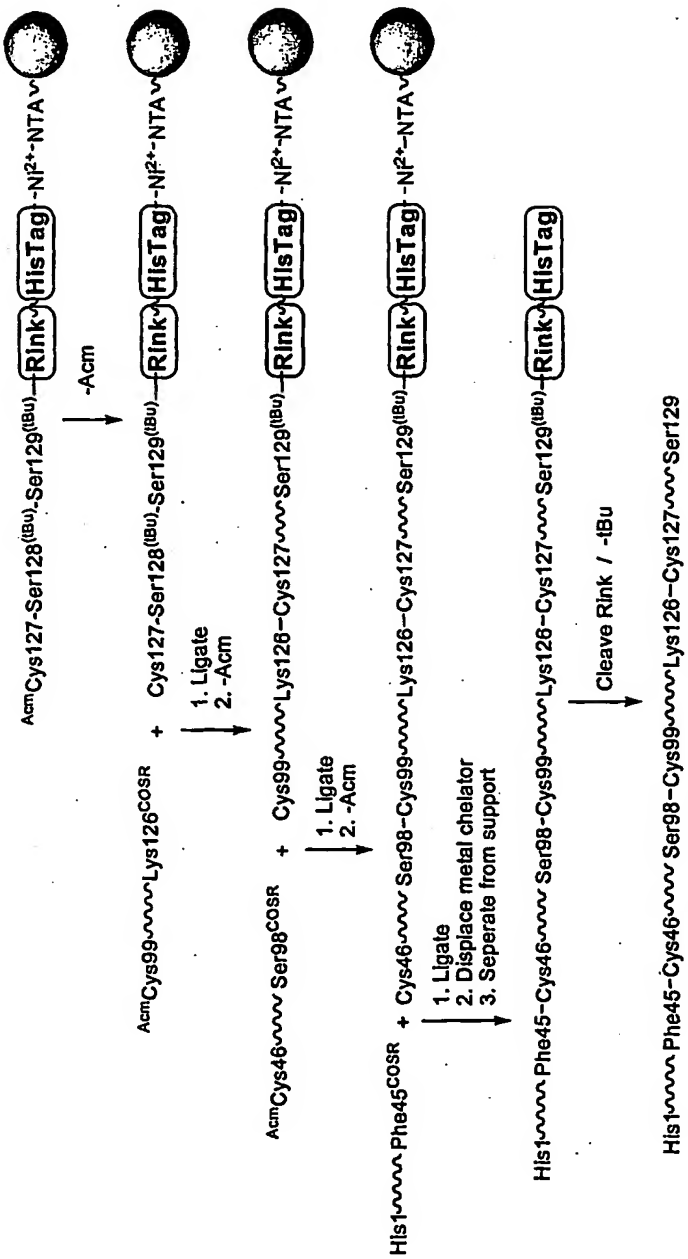

AcmCys127-Ser128(tBu)-Ser129(tBu)-Rink-HisTag-Ni2+-NTA
-Acm
AcmCys99-Lys126COSR + Cys127-Ser128(tBu)-Ser129(tBu)-Rink-HisTag-Ni2+-NTA
1. Ligate
2. -Acm
AcmCys46-Ser98COSR + Cys99-Lys126-Cys127-Ser129(tBu)-Rink-HisTag-Ni2+-NTA
1. Ligate
2. -Acm
His1-Phe45COSR + Cys46-Ser98-Cys99-Lys126-Cys127-Ser129(tBu)-Rink-HisTag-Ni2+-NTA
1. Ligate
2. Displace metal chelator
3. Seperate from support
His1-Phe45-Cys46-Ser98-Cys99-Lys126-Cys127-Ser129(tBu)-Rink-HisTag
Cleave Rink / -tBu
His1-Phe45-Cys46-Ser98-Cys99-Lys126-Cys127-Ser129

FIGURE 4

FIG.4A

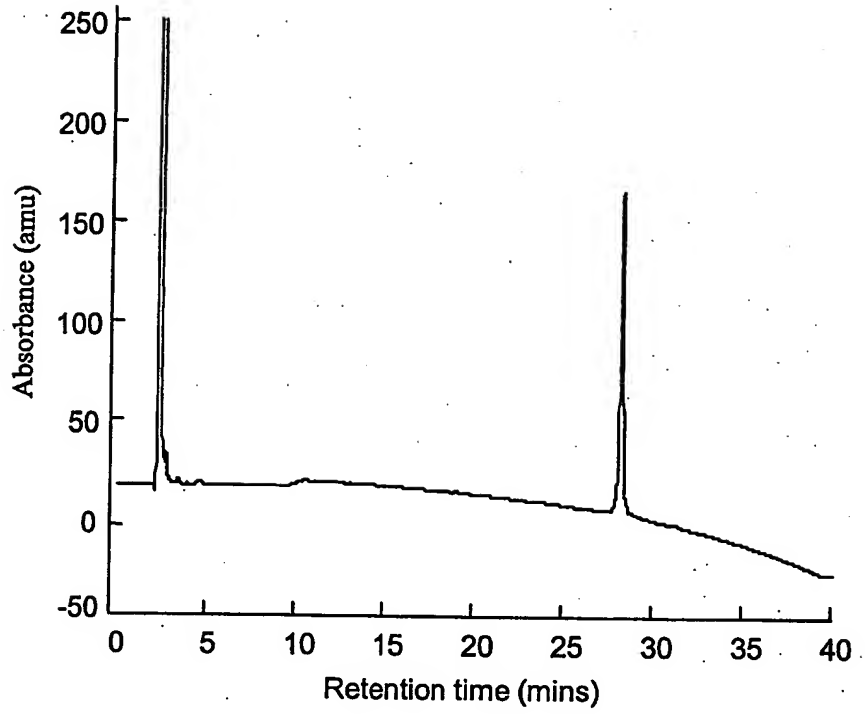


FIG.4B